UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

EFEITOS DO CLORETO DE MERCÚRIO E DO CLORETO DE ZINCO SOBRE PARÂMETROS RENAIS E HEPÁTICOS EM RATAS LACTANTES E NÃO-LACTANTES

TESE DE DOUTORADO

ALEXANDRE MARAFON FAVERO

Santa Maria, RS, Brasil

2011

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por

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS),
como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

Orientadora: Maria Ester Pereira

Santa Maria, RS, Brasil

2011

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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Elaborada por **Alexandre Marafon Favero**
como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 08 de abril de 2011.

AGRADECIMENTOS

À Profª. Drª. Maria Ester Pereira, por ter me aceito como orientado, pela amizade, conhecimentos transmitidos e pela atenção dispensada durante o desenvolvimento do meu trabalho. “Tu sabes que serei sempre grato por tudo que fizeste por mim”.

À Profª. Drª. Carina, pelos ensinamentos e por ter me auxiliado em toda a parte experimental deste trabalho.

Aos colegas de laboratório, Rafael, Lucélia, Vitor, Tânia e Aline que, além de colegas, demonstraram-se grandes amigos. Agradeço-os pelo convívio e conhecimento compartilhado ao longo desse período.

Às alunas de iniciação científica Naglezi, Fernanda e, em especial, à Cláudia pela dedicação, pelo companheirismo e por ter assumido diversas responsabilidades nos dois últimos anos de realização deste trabalho.

À Profª. Sônia, do Departamento de Patologia, pela contribuição nos estudos histopatológicos.

Aos meus pais e a minha irmã, que, embora distantes, sempre me incentivaram para o aperfeiçoamento do meu conhecimento e acreditaram em meu trabalho.

À Si, em especial, por todo seu amor e companheirismo. “Obrigado pelo carinho e pela força em todos os momentos”.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica pela possibilidade de realização deste curso.

Aos professores e aos colegas do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica que de alguma maneira contribuíram para a minha formação científica.

Aos funcionários do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica pela dedicação e competência com que realizam os seus trabalhos.

Ao CNPq pela bolsa de estudos e às demais agências de fomento, em especial à CAPES, pelo auxílio financeiro durante a realização deste trabalho.

Aos colegas da CAPES que me incentivaram para finalização deste trabalho.

Enfim, agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

RESUMO

Tese de doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

EFEITOS DO CLORETO DE MERCÚRIO E DO CLORETO DE ZINCO SOBRE PARÂMETROS RENAIS E HEPÁTICOS EM RATAS LACTANTES E NÃO-LACTANTES

Autor: Alexandre Marafon Favero
Orientadora: Maria Ester Pereira
Local e data da defesa: Santa Maria, 08 de abril de 2011.

O objetivo deste estudo foi comparar os efeitos da exposição ao cloreto de mercúrio ($HgCl_2$) sobre parâmetros renais e hepáticos em ratas adultas não-lactantes e ratas lactantes e seus filhotes, e avaliar o possível efeito preventivo do zinco (Zn), administrado na forma de cloreto de zinco ($ZnCl_2$), sobre os efeitos nefro e hepatotóxicos causados pela exposição ao mercúrio inorgânico. As ratas lactantes e não-lactantes foram pré-expostas a uma dose diária de $ZnCl_2$ (27 mg/kg/dia; s.c.) ou solução salina 0,9% durante cinco dias. Nos cinco dias subsequentes, as ratas foram expostas a uma dose diária de $HgCl_2$ (5 mg/kg/dia; s.c.) ou salina 0,9%. A exposição das ratas lactantes aos metais iniciou-se no 3º dia de lactação. Os filhotes foram expostos aos metais exclusivamente via leite materno. Os animais foram observados diariamente quanto aos sinais de toxicidade e mortalidade. O consumo de água e de ração das ratas lactantes e não-lactantes foi monitorado diariamente durante o período de exposição aos metais. Os animais foram eutanaziados 24 horas após a administração da última dose de $HgCl_2$. Amostras de sangue, rim e fígado foram retiradas para a análise dos seguintes parâmetros: atividade da enzima d-aminolevulinato desidratase (d-ALA-D); parâmetros bioquímicos indicativos de toxicidade renal (níveis plasmáticos de uréia e creatinina) e hepática (atividade das enzimas AST, ALT e LDH plasmáticas) e os níveis de metais (Hg e Zn) nos tecidos estudados. Nas ratas não-lactantes, a taxa de sobrevivência, o consumo de ração, os pesos do corpo e dos rins, a atividade da enzima d-ALA-D sanguínea e renal, os níveis plasmáticos de uréia e creatinina, a atividade das enzimas AST e ALT plasmáticas, a histologia do tecido renal, os níveis de zinco no sangue e os níveis de mercúrio no sangue, rins e fígado foram significativamente alterados pela exposição ao $HgCl_2$. A exposição prévia ao $ZnCl_2$ preveniu alguns dos efeitos induzidos pelo mercúrio, tais como: a diminuição na taxa de sobrevivência, o aumento nos níveis plasmáticos de uréia e creatinina, a inibição da atividade da enzima d-ALA-D sanguínea (parcialmente) e renal, o aumento na atividade da AST (parcialmente) e a diminuição dos níveis sanguíneos de zinco. Por outro lado, o $ZnCl_2$ não foi capaz de prevenir os efeitos do mercúrio sobre a diminuição do consumo de ração e dos pesos corporal e renal, a inibição da atividade da ALT, as alterações histológicas e os níveis de mercúrio nos tecidos. Nas lactantes, o consumo de ração, os pesos do corpo e dos rins, a atividade das enzimas d-ALA-D sanguínea e hepática e ALT plasmática, os níveis de zinco no sangue e os níveis de mercúrio no sangue e nos rins foram significativamente alterados pela exposição ao $HgCl_2$. A pré-exposição ao $ZnCl_2$ não preveniu nenhuma das alterações bioquímicas e fisiológicas induzidas pela exposição ao $HgCl_2$. Além disso, essa pré-exposição potencializou o acúmulo de mercúrio nos tecidos renal e hepático e

induziu o aparecimento de alterações histológicas no fígado, as quais não foram observadas nas ratas lactantes expostas exclusivamente ao $HgCl_2$. Em relação aos filhotes, o ganho de peso corporal, os pesos absolutos de rins e fígado e o acúmulo de mercúrio nesses tecidos foram significativamente alterados pela exposição indireta ao metal tóxico via leite materno. Nenhuma dessas alterações foram prevenidas pela exposição prévia das lactantes ao $ZnCl_2$. Este estudo demonstrou, pela primeira vez, que as ratas lactantes expostas ao $HgCl_2$ apresentam respostas bioquímicas distintas em relação as ratas adultas não-lactantes quando analisados parâmetros renais e hepáticos de toxicidade. Além disso, estes resultados demonstram que o mercúrio é transferido aos filhotes via leite materno e que os níveis de mercúrio disponíveis não são suficientes para alterar os parâmetros bioquímicos analisados. O papel preventivo do $ZnCl_2$ sobre a toxicidade renal induzida pelo $HgCl_2$ nas ratas não-lactantes sugere efetivamente que ele serve como alternativa promissora no tratamento preventivo dos casos de exposição ao mercúrio inorgânico. Entretanto, uma vez que a pré-exposição ao $ZnCl_2$ potencializou os efeitos do $HgCl_2$ sobre os níveis de mercúrio em rim e fígado e induziu alterações histológicas no tecido hepático de ratas lactantes, sugere-se que o $ZnCl_2$ deva ser usado com cautela durante o período da lactação e que mais estudos são necessários para certificar-se da segurança de seu uso nesse período.

Palavras-chave: cloreto de mercúrio; cloreto de zinco; período de lactação; ratos lactentes; d-aminolevulinato desidratase.

ABSTRACT

Thesis of Doctor's Degree
Graduate Program in Biological Science: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EFFECTS OF MERCURY CHLORIDE AND ZINC CHLORIDE ON RENAL AND HEPATIC PARAMETERS IN LACTATING AND NON-LACTATING RATS

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Date and place of the defense: Santa Maria, April 8, 2011.

The aim of this study was to compare the effects of mercuric chloride ($HgCl_2$) on renal and hepatic parameters in adult non-lactating and lactating rats and their pups and to assess the potential preventive role of Zn, given as zinc chloride ($ZnCl_2$), on the nephrotoxic and hepatotoxic effects caused by exposure to inorganic mercury. Non-lactating and lactating rats were pre-exposed to a daily dose of $ZnCl_2$ (27 mg/kg/day; s.c.) or saline 0.9% during five consecutive days and to a daily dose of $HgCl_2$ (5 mg/kg/day; s.c.) or saline 0.9% for the five subsequent days. The exposure of lactating rats to metals began on day 3 of lactation. Suckling pups were exposed to metals exclusively through maternal milk. Animals were observed daily throughout the study for signs of toxicity and mortality. Water and food consumption of lactating and non-lactating rats were monitored daily during the entire period of exposure to metals. Animals were euthanized 24 h after the last dose of $HgCl_2$ and tissue samples were collected (blood, kidney and liver) to analyze the following parameters: d-aminolevulinic acid dehydratase (d-ALA-D) activity; biochemical parameters indicative of renal (plasma urea and creatinine levels) and hepatic (plasma AST, ALT and LDH activities) toxicity and the metal levels (Hg and Zn) in all tissues studied. In non-lactating rats, the survival rate; food consumption; body and kidney weights; blood and renal d-ALA-D activity; plasma urea and creatinine levels; plasma ALT and AST activities; renal histology; blood Zn levels and blood, kidney and liver Hg levels were significantly affected by $HgCl_2$ exposure. Previous exposure to $ZnCl_2$ prevented some of the effects of mercury, such as: decrease in survival rate, increase in plasma urea and creatinine levels, inhibition in blood (partially) and renal d-ALA-D activity, the increase in plasma AST (partially) activity and the decrement in blood Zn levels. In contrast, $ZnCl_2$ was unable to prevent the effects of mercury on the decrease in food consumption and in body and kidney weights, inhibition of plasma ALT activity, renal histological alterations and on the increased Hg levels in tissues. In lactating rats, food consumption, body and kidney weights, blood and hepatic d-ALA-D activity, plasma ALT activity and Hg levels in blood and kidneys were significantly modified by $HgCl_2$ exposure. Previous exposure to $ZnCl_2$ was not able to prevent any physiological and biochemical changes induced by $HgCl_2$ exposure. Moreover, the pre-exposure to $ZnCl_2$ potentiated the effects of $HgCl_2$ exposure on retention of Hg in renal and hepatic tissues and induced histological alterations in the liver (which were not observed when lactating rats were exposed to $HgCl_2$ alone). In pups, body weight gain, absolute kidney and liver weights and retention of Hg in these tissues were significantly altered by indirect

exposure to heavy metal through maternal milk. None of these changes were prevented by pre-exposure of their mothers to $ZnCl_2$. Taken together, this study showed for the first time that lactating rats exposed to $HgCl_2$ presented distinct biochemical responses comparing to non-lactating rats when renal and hepatic parameters were evaluated. Furthermore, these results showed that mercury is transferred to the pups through maternal milk and that mercury levels available to pups were not sufficient to induce any change in biochemical parameters evaluated. The preventive effect of $ZnCl_2$ on renal toxicity induced by $HgCl_2$ in non-lactating rats suggests effectively that it serves as a promising alternative for the preventive treatment of inorganic mercury poisoning cases; however, since pre-exposure to $ZnCl_2$ potentiated the effects of $HgCl_2$ on mercury levels in kidney and liver and induced histological changes in hepatic tissue of lactating rats, we suggest that $ZnCl_2$ should be used with caution during lactation and that more studies are necessary to ensure the safety of its use in this period.

Keywords: mercuric chloride; zinc chloride; lactation period; suckling pup rats; d-aminolevulinic acid dehydratase.

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LISTA DE REDUÇÕES (ABREVIATURAS, SIGLAS E SÍMBOLOS)

AChE: acetilcolinesterase (*acetylcholinesterase*);

AAS: espectrofotometria de absorção atômica (*atomic absorption spectrophotometry*);

d-ALA: ácido d-aminolevulínico (d-*aminolevulinic acid*);

d-ALA-D: d-aminolevulinato desidratase (d-*aminolevulinic acid dehydratase*) (=PBG-Sintase);

ALT: alanina aminotransferase (*alanine aminotransferase*);

ANOVA: análise de variância (*analysis of variance*);

AST: aspartato aminotransferase (*aspartate aminotransferase*);

ATC: acetiltiocolina iodada (*acetylthiocoline iodide*);

b.w.: *body weight*

DNA: ácido desoxirribonucléico (*deoxyribonucleic acid*);

DTNB: ácido 5,5'-ditio*bis*-2-nitrobenzóico [*5,5'-dithio-bis(2-nitrobenzoic acid)*];

E.C.: Comissão de Enzimas *(Enzyme Comission)*;

HCl: ácido clorídrico (*chloridric acid*);

H&E: hematoxilina e eosina (*haematoxylin and eosin*);

$HgCl_2$: cloreto mercúrico; cloreto de mercúrio II (*mercuric chloride*);

HNO_3: ácido nítrico (*nitric acid*);

H_2SO_4: ácido sulfúrico (*sulfuric acid*);

K_2HPO_4: fosfato de potássio dibásico (*potassium phosphate dibasic*);

KH_2PO_4: fosfato de potássio monobásico (*potassium phosphate monobasic*);

LD: *lactational day*;

LDH: lactato desidrogenase (*lactate dehydrogenase*);

MT: metalotioneína(s);

n: número de repetições;

NaCl: cloreto de sódio (*sodium chloride*);

NAD^+: dinucleotídeo de nicotinamida adenina (forma oxidada) [*nicotinamide adenine dinucleotide (oxidized form)*];

NADH: dinucleotídeo de nicotinamida adenina (forma reduzida) [*nicotinamide adenine dinucleotide (reduced form)*];

NaOH: hidróxido de sódio (*sodium hidroxide*);

p: nível de significância;

P: nível de significância;

PAS: *Periodic-acid Schiff*;

PBG: porfobilinogênio (*porphobilinogen*);

PBG-sintase: porfobilinogênio sintase (*porphobilinogen synthase*) (=d-ALA-D);

pH: potencial hidrogeniônico;

rpm: rotações por minuto;

Sal: salina (*saline*);

s.c.: subcutânea; subcutaneamente (*subcutaneously*);

S.E.M.: erro padrão da média (*standard error of mean*);

SH: grupamento(s) sulfidrílico(s);

TCA: ácido tricloroacético (*trichloroacetic acid*);

U: unidade;

w/v: peso/volume (*weight/volume)*;

$ZnCl_2$: cloreto de zinco (*zinc chloride*).

SUMÁRIO

APRESENTAÇÃO

No item **INTRODUÇÃO** está descrita uma breve introdução sobre a tese. No final deste item estão apresentados os objetivos, geral e específicos, desta tese.

No item **REVISÃO BIBLIOGRÁFICA** está descrita uma sucinta revisão sobre os temas trabalhados nesta tese.

Os **RESULTADOS** estão dispostos na forma de manuscritos submetidos à publicação. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se nos próprios manuscritos e representam a íntegra deste estudo.

No item **DISCUSSÃO** estão apresentados as interpretações e comentários gerais sobre os manuscritos científicos aqui apresentados.

No item **CONCLUSÕES** são apresentadas as conclusões gerais do presente trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** apresentadas no final da tese referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO.**

No item **APÊNDICES** são apresentados resultados adicionais de experimentos realizados durante o curso de doutorado, mas não incluídos no corpo da tese.

1. INTRODUÇÃO

O mercúrio (Hg) ocorre naturalmente no ambiente em diferentes espécies químicas (elementar, orgânica e inorgânica) com solubilidade, reatividade e toxicidade distintas, causando diferentes impactos no ecossistema e na saúde do homem (Klaassen, 1996). As formas orgânicas de mercúrio afetam principalmente o sistema nervoso central (Fredriksson *et al.*, 1992; Risher *et al.*, 2003; Counter & Buchanan, 2004; Yoshida *et al.*, 2005), enquanto que as formas inorgânicas, cujo principal representante é o cloreto de mercúrio ($HgCl_2$), causam toxicidade renal (Clarkson, 1997; Herak-Kramberger & Sabolic, 2001).

A exposição a compostos de mercúrio pode causar toxicidade aos organismos durante os períodos iniciais do desenvolvimento (gestação e lactação). Sabe-se que as formas orgânicas desse metal são mais prejudiciais ao desenvolvimento do feto, uma vez que elas atravessam com mais facilidade a barreira placentária do que as formas inorgânicas (Kajiwara *et al.*, 1996). Por outro lado, estudos realizados durante o período de lactação demonstraram que as formas inorgânicas de mercúrio são excretadas mais eficientemente no leite materno do que as formas orgânicas (Sundberg *et al.*, 1991a; 1991b; 1998; Oskarsson *et al.*, 1996). Portanto, estudos toxicológicos envolvendo a exposição materna a compostos inorgânicos de mercúrio durante o período neonatal são extremamente importantes uma vez que durante este período os organismos em desenvolvimento são mais sensíveis aos efeitos da exposição a agentes externos (Barone *et al.*, 2000; Rice & Barone, 2000; Mendola *et al.*, 2002; Morgane *et al.*, 2002).

Estudos prévios demonstraram que durante o período de lactação ocorre uma diminuição da meia-vida biológica do mercúrio (Greenwood *et al.*, 1978; Magos, 1980; Prester *et al.*, 1994; 1997), o que pode levar a uma modificação dos efeitos tóxicos deste metal durante este período. Embora existam na literatura diversos trabalhos demonstrando os efeitos tóxicos das formas inorgânicas de mercúrio e a distribuição deste metal entre os órgãos, não existem estudos que demonstrem diferença na susceptibilidade aos danos causados pela exposição ao mercúrio inorgânico no período de lactação, por isso a importância de se comparar os efeitos deste metal em organismos adultos (lactantes e não-lactantes) expostos durante este período.

1.1 Objetivos

O presente estudo tem como objetivo geral comparar a sensibilidade de ratas lactantes e não-lactantes expostas ao cloreto de mercúrio quanto a parâmetros renais e hepáticos e investigar a eficiência da pré-exposição ao zinco em prevenir os efeitos tóxicos do mercúrio.

Dessa forma, os objetivos específicos são:

- investigar os efeitos da exposição ao cloreto de mercúrio sobre o ganho de peso corporal, o peso dos órgãos, o consumo de água e ração de ratas lactantes e não-lactantes;
- analisar os níveis de deposição de mercúrio em sangue, rins e fígado como consequência da exposição ao cloreto de mercúrio;
- avaliar os efeitos da exposição ao cloreto de mercúrio sobre a atividade da enzima d-aminolevulinato desidratase (d-ALA-D) de sangue, rins e fígado;
- investigar os efeitos da exposição ao cloreto de mercúrio sobre parâmetros marcadores de função renal e hepática;
- avaliar possíveis alterações teciduais em rins e fígado devido à exposição ao cloreto de mercúrio;
- verificar o efeito da pré-exposição ao zinco sobre todos estes parâmetros; e
- investigar os efeitos dos metais sobre os filhotes expostos via leite materno.

2. REVISÃO BIBLIOGRÁFICA

2.1. Mercúrio

O mercúrio (Hg) é um elemento sem função fisiológica no organismo humano, sendo tóxico aos seres vivos em geral nas suas diferentes formas físico-químicas (elementar, orgânica e inorgânica). As diferentes espécies de mercúrio apresentam solubilidade, reatividade e toxicidade diferentes e, consequentemente, comportam-se de maneira distinta no meio ambiente (Klaassen, 1996). O mercúrio elementar (ou vapor de mercúrio ou mercúrio metálico) possui estado de oxidação zero (Hg^0) e é volátil (Salgado, 1996). As formas orgânicas de mercúrio, das quais o metilmercúrio (CH_3Hg^+) é a mais tóxica, originam-se a partir da ligação covalente do mercúrio a átomos de carbono e afetam principalmente o sistema nervoso central (Fredriksson *et al.*, 1992; Risher *et al.*, 2003; Counter & Buchanan, 2004; Yoshida *et al.*, 2005). As formas inorgânicas, também chamadas de sais de mercúrio, existem em dois estados de oxidação: sais mercurosos monovalentes Hg(I) ou mercúricos bivalentes Hg(II) (UNEP, 2002; Klaassen, 1996). As formas inorgânicas de mercúrio, cujo principal representante é o cloreto de mercúrio ($HgCl_2$) causam, principalmente, toxicidade renal (Clarkson, 1997; Herak-Kramberger & Sabolic, 2001).

Na sua forma natural, este metal apresenta-se como mercúrio elementar, podendo ser lançado na atmosfera por meio da emissão de gases vulcânicos, da degradação da crosta terrestre e da evaporação de corpos hídricos (WHO, 1991; Goyer, 1996). Além disso, o mercúrio pode ser introduzido no ambiente por inúmeras fontes antropogênicas (WHO, 1989). Durante décadas o mercúrio foi utilizado para fins industriais o que resultou no aumento da contaminação ambiental (Salgado, 1996). Apesar da redução de seu uso nas últimas décadas, este metal não-essencial continua contaminando rios e solos, pois ainda é utilizado em processos industriais e, principalmente, na mineração (Lacerda & Pfeiffer, 1992). Atualmente, a população em geral corre o risco de se expor ao mercúrio presente em amálgamas dentários, termômetros, medidores de pressão arterial, baterias e lâmpadas fluorescentes (Clarkson *et al.*, 2003). No Brasil, em especial na região amazônica, a principal fonte de exposição ao mercúrio ocorre em garimpos, onde este metal é utilizado no processo de amalgamação das partículas de ouro (Lacerda & Pfeiffer, 1992; Pinheiro *et al.*, 2000).

Após esta ligação, o amálgama é queimado para separação do ouro, o que promove a volatilização do mercúrio; o restante do mercúrio é lançado diretamente em rios e solos (Lacerda & Pfeiffer, 1992).

Segundo estimativas das emissões de mercúrio atmosférico no Brasil, o garimpo contribui com mais de 65% do total das emissões antrópicas (Lacerda & Marins, 1997). Na atmosfera, o mercúrio metálico é oxidado pelo ozônio, formando o cloreto de mercúrio. Este sal de mercúrio inorgânico deposita-se na água e no solo, onde pode ser metilado por bactérias anaeróbicas, formando metilmercúrio, ou se volatilizar, retornando ao ambiente (Bisinoti & Jardim, 2004). O mercúrio orgânico acumula-se na cadeia alimentar aquática através do processo denominado bioamplificação, ou seja, a concentração do metal aumenta em organismos vivos ao passar de níveis tróficos inferiores (herbívoros) para níveis superiores (carnívoros). Portanto, os peixes consumidos pela população em geral, especialmente a ribeirinha, podem possuir uma alta concentração de mercúrio em sua forma orgânica (Boening, 2000; UNEP, 2002, Wasserman *et al.*, 2003). Um exemplo desta situação é relatado em um trabalho realizado na região amazônica brasileira, onde peixes consumidos pela população local apresentaram níveis de mercúrio total acima do limite recomendável para o consumo humano pela Organização Mundial da Saúde (OMS) (Akagi *et al.*, 1995).

2.1.1. Toxicidade do mercúrio inorgânico

Os efeitos tóxicos da exposição ao mercúrio inorgânico têm sido bastante estudados. Essa forma de mercúrio exerce seus efeitos em diferentes órgãos, tecidos e células, sendo conhecido principalmente por induzir danos ao sistema renal. No entanto, sabe-se também que o mercúrio inorgânico pode causar danos hepáticos e ao sistema nervoso central. É importante ressaltar que no presente estudo serão enfatizados os efeitos do mercúrio inorgânico (cloreto de mercúrio) sobre os sistemas renal e hepático.

2.1.1.1. Nefrotoxicidade

A principal função dos rins é a excreção de substâncias tóxicas oriundas do metabolismo. Além disso, os rins também desempenham um papel importante na

homeostase corporal, regulando o volume de líquido extracelular, o equilíbrio eletrolítico e a pressão arterial (Hodgson & Levi, 2004).

A nefrotoxicidade causada pela exposição a metais tóxicos deve-se, em parte, ao fato de que a eliminação urinária é a principal rota de excreção destes (Fowler, 1993). Os rins constituem o alvo primário da toxicidade e acúmulo do mercúrio inorgânico (Clarkson *et al.*, 2003). O mercúrio é reabsorvido e acumulado, predominantemente, nas células dos túbulos proximais (ATSDR, 1994; Zalups & Lash, 1994). Como os íons mercúricos são altamente reativos, combinam-se intracelularmente com grupamentos sulfidrílicos causando destruição ou inativação de proteínas e enzimas essenciais para uma função renal adequada. Este metal também promove estresse oxidativo, peroxidação lipídica e disfunção mitocondrial (Zalups, 2000). A toxicidade renal causada pelo mercúrio é constatada pelo aumento nos níveis sanguíneos de uréia e creatinina, tanto em ratos jovens (Peixoto & Pereira, 2007; Franciscato *et al*., 2011) como em animais adultos (Sener *et al*., 2007). De fato, a análise dos níveis sanguíneos de uréia e creatinina é utilizada clinicamente para o diagnóstico de danos renais (Hodgson & Levi, 2004). A uréia é o principal produto do catabolismo das proteínas, servindo como um mecanismo de excreção da amônia proveniente das reações de desaminação. A creatinina é formada durante o metabolismo normal da musculatura a partir da degradação da fosfocreatina, sendo geralmente produzida em uma taxa constante no organismo (Finco, 1997). Fisiologicamente, estes metabólitos são excretados pelos rins, portanto, o aumento de seus níveis no sangue indica um dano na função renal (Ravel, 1997).

2.1.1.2. Hepatotoxicidade

O fígado é o maior órgão do corpo humano e desempenha um papel central no metabolismo. A fisiologia hepática é altamente especializada no cumprimento de diversas funções conhecidas, tais como: metabólicas, excretoras, secretoras, armazenamento, protetoras, circulatórias e de coagulação sanguínea. As doenças hepáticas são um problema de saúde pública, sendo que a evolução das mesmas inicia-se com a esteatose, hepatite, fibrose, cirrose, podendo evoluir até o carcinoma hepatocelular (Loguercio e Frederico, 2003; Vitaglione *et al*., 2004). A confirmação do dano hepático é realizada por meio de exames específicos, como a dosagem da atividade

enzimática das aminotransferases [alanina (ALT) e aspartato (AST) aminotransferase], lactato desidrogenase (LDH), fosfatase alcalina, γ-glutamil-transferase, entre outras. Estas são enzimas de função intracelular presentes em vários tecidos e sua presença no sangue é uma consequência da liberação anormal para a circulação (Devlin, 1997).

A hepatotoxicidade causada pelo mercúrio inorgânico aparece refletida pelo aumento sanguíneo de enzimas presentes no interior dos hepatócitos, tais como a ALT, a AST e a LDH (El-Demerdash, 2001; Kumar *et al*., 2005; Sener *et al*., 2007; Sharma *et al*., 2007), utilizadas como marcadores de dano hepatocelular (Meyer *et al*., 1992). As aminotransferases (AST e ALT) são enzimas intracelulares que catalisam a transferência reversível do grupo amino de um aminoácido para um aceptor a-cetoácido, resultando na formação de um novo aminoácido e um novo a-cetoácido. A LDH catalisa a reação reversível da conversão de piruvato em lactato. Estas enzimas estão presentes em vários tecidos envolvidos no metabolismo de proteínas e carboidratos, incluindo o tecido hepático (Devlin, 1997). Recentemente, foi demonstrado que a atividade da LDH não é alterada e a da ALT é inibida em soro de ratos jovens expostos ao $HgCl_2$ (Peixoto & Pereira, 2007).

As formas inorgânicas do mercúrio também podem causar alterações histopatológicas tais como vacuolização, cariorrexia, cariólise, picnose e necrose centrolobular (Kumar *et al*., 2005; Sharma *et al*., 2000; 2002; 2007). Além disso, foi demonstrado que os efeitos hepatotóxicos do $HgCl_2$ parecem estar associados ao dano mitocondrial, por inibir a fosforilação oxidativa, levando a uma falha na produção de energia e, consequentemente, a uma lesão hepatocelular letal (Palmeira & Madeira, 1997).

2.2. Zinco

O zinco é um metal essencial relacionado com funções vitais nos tecidos dos mamíferos, estando presente em inúmeras proteínas e enzimas, envolvido em funções estruturais, catalíticas e regulatórias (Chan *et al.*, 2002; Maret, 2005; Mathie *et al.*, 2006). A replicação e a transcrição do ácido desoxirribonucléico (DNA) e a síntese protéica são processos que representam o papel essencial do zinco na regulação da proliferação e diferenciação celulares (Eckhert & Hurley, 1977; Cherian *et al.*, 2003)

sendo este elemento, portanto, considerado essencial para o crescimento e desenvolvimento. Sua essencialidade também é evidente considerando-se que ele é imprescindível a uma variedade de funções bioquímicas e fisiológicas, nas quais estão envolvidas várias enzimas e proteínas que o requerem (Vallee, 1995; Rofe *et al.*, 2000; Zatta *et al.*, 2003; Takeda *et al.*, 2004a, 2004b, 2005). Além disso, o zinco é necessário para o sistema imunológico, o metabolismo intermediário, o metabolismo e a reparação do DNA, a reprodução e o processo de apoptose (Maret & Sandstead, 2006). A manutenção das concentrações adequadas de zinco é de suma importância de modo a manter todos esses eventos sob um controle rígido.

No plasma, o zinco está presente em concentrações aproximadas de 15 µM, sendo transportado ligado à albumina (Mathie *et al*., 2006), enquanto que nos tecidos encontra-se ligado a metaloproteínas, tais como as metalotioneínas (MT), e sua concentração é variável. A homeostase do metal é realizada por meio de proteínas sensoras e transportadoras de membranas, as quais regulam a entrada e a saída de zinco na célula (Chimienti *et al*., 2003). As MT participam do transporte deste metal entre as organelas, mantendo o zinco disponível em concentrações adequadas (Mason *et al.*, 1981; Cuajungco & Lees, 1997; Rofe *et al.*, 2000; Maret, 2005). Diversos autores já demonstraram que alguns metais possuem a capacidade de induzir a síntese de proteínas ligantes de metal e MT (Eaton *et al.*, 1980; Goering & Fowler, 1987; Pedersen *et al*., 1998) e, entre eles, o zinco é classificado como o melhor indutor destas proteínas (Eaton *et al.*, 1980; Bracken & Klaassen, 1987). Vários estudos relataram os efeitos benéficos do zinco em relação aos danos induzidos por alguns agentes como o mercúrio (Gale, 1984; Zalups & Cherian, 1992; Counter & Buchanan, 2004), o cádmio (Tang *et al.*, 1998; Brzóska *et al.*, 2001), o cobre (Brewer *et al.*, 1998) e organofosforados (Goel *et al.*, 2005, 2006).

Estudos recentes demonstraram que o zinco pode ser considerado um agente protetor contra a toxicidade do $HgCl_2$. Quando utilizado como um pré-tratamento, o zinco é capaz de prevenir a diminuição no ganho de peso corporal, evitar a inibição da enzima d-ALA-D renal e hepática (Peixoto *et al*., 2003), bem como evitar a insuficiência renal causada pela exposição ao $HgCl_2$ (Peixoto & Pereira, 2007). Estudos sugerem que os efeitos preventivos do zinco podem ser parcialmente atribuídos à síntese de MT induzida por este metal (Peixoto *et al*., 2003; 2007a), já que ele é considerado o melhor indutor desta proteína (Eaton *et al*., 1980; Bracken & Klaassen,

1987). As MT possuem uma grande afinidade por metais devido ao alto conteúdo de resíduos de cisteína presentes em sua estrutura (Chan *et al*., 2002), por isso desempenham um importante papel no transporte e estocagem de metais essenciais bem como na detoxificação de metais não essenciais (Hidalgo *et al*., 2001).

O zinco é um elemento essencial requerido para o crescimento e desenvolvimento normal (Krebs, 1999). O nível desse elemento é maior no colostro (leite secretado pela maioria dos mamíferos nos primeiros dias de lactação) e diminui progressivamente ao longo do período de lactação (Kirksey *et al*., 1979; Moore *et al*., 1984; Krebs *et al*., 1985; Karra *et al*., 1988; Moser-Veillon & Reynolds, 1990; Krebs *et al*., 1995). Estudos que examinaram o efeito da suplementação materna com zinco sobre as concentração desse metal no leite obtiveram resultados distintos: dois estudos demonstraram que os níveis de zinco no leite são influenciados pela ingestão materna de zinco (Krebs *et al*., 1985; Karra *et al*., 1988), enquanto outros demonstraram a inexistência de correlação entre a ingestão de zinco e a concentração deste elemento no leite materno (Kirksey *et al*., 1979; Moore *et al*., 1984; Moser-Veillon & Reynolds, 1990; Krebs *et al*., 1995). Em um estudo recente, Krebs (1999) demonstrou que as concentrações de zinco no leite não aumentam em resposta ao aumento da ingestão de zinco se o *status* de zinco materno estiver adequado. De acordo, foi demonstrado que as concentrações de zinco no leite humano podem responder à suplementação alimentar apenas quando o estado nutricional da lactante é pobre em zinco (Fomon, 1993).

2.3. Período de lactação

A lactação é o fenômeno fisiológico neuroendócrino de produção de leite pelas fêmeas dos mamíferos no período pós-parto, uma característica única desta classe (Carvalho & Tamez, 2002). O processo de lactação é imprescindível para assegurar a sobrevivência do recém-nascido, pois é uma das maneiras mais eficientes de atender os aspectos nutricionais, imunológicos e psicológicos no período inicial de desenvolvimento (Ichisato & Shimo, 2001). A lactação representa um estado de alta demanda energética e de nutrientes para a produção de leite (IOM, 2002). Tal demanda pode ser alcançada através dos estoques maternos e do aumento da ingestão de nutrientes. Além disso, mudanças fisiológicas e hormonais neste período afetam o metabolismo e a regulação da homeostase de nutrientes com a finalidade de garantir

uma eficiente utilização dos mesmos pelo organismo materno e, consequentemente, uma eficiente transferência destes para o leite (IOM, 2002).

A lactação consiste em uma série de etapas morfo-fisiológicas no processo de diferenciação, pelas quais as células mamárias são convertidas de um estado não-secretor para um estado secretor, sendo mantido através de estímulos de ejeção do leite e controlado pelos hormônios lactogênicos (Martins *et al.*, 2010). A lactação é um processo complexo no qual ocorrem importantes alterações hormonais, fisiológicas e metabólicas (Martins *et al.*, 2010). Após o parto, a diminuição dos níveis de estrógeno e progesterona e a elevação dos níveis de prolactina estimulam o início da secreção de leite pela glândula mamária (Picciano, 2003). A ocitocina, um hormônio produzido no hipotálamo e armazenado na neuro-hipófise, desempenha um papel essencial na ejeção do leite. A sucção das mamas pelo lactente resulta na transmissão de impulsos sensitivos por nervos somáticos dos mamilos até a medula espinhal e, daí, para o hipotálamo, promovendo a liberação da ocitocina na corrente sanguínea. A ocitocina é, então, transportada pelo sangue para as glândulas mamárias onde estimula a contração das células mioepiteliais que revestem o lúmen alveolar, forçando a descida do leite para os ductos (Kensinger, 1998; Guyton, 2006). A ativação dos receptores neurais libera também a prolactina, o hormônio do crescimento, o hormônio adrenocorticotrópico e o hormônio estimulante da tireóide pela hipófise anterior (Klopfenstein *et al.*, 2006), além da gastrina e do glucagon (Algers, 1993) a fim de regular a síntese do leite (galactopoiese) pelas células epiteliais mamárias.

Juntamente com as alterações hormonais, as lactantes apresentam adaptações metabólicas importantes no período pós-parto. Chatwin *et al.* (1969) demonstraram que existem diferenças entre ratas lactantes e não-lactantes em relação ao sistema cardiovascular. O débito cardíaco é maior nas ratas lactantes e alguns tecidos, em particular as glândulas mamárias, o fígado e o trato gastrointestinal, recebem um correspondente fluxo sanguíneo maior. O aumento no débito cardíaco associado às alterações no fluxo sanguíneo nos tecidos periféricos provavelmente ocorre quando a demanda por leite materno pelos filhotes é maior do que a capacidade do tecido mamário em sintetizá-lo em quantidade suficiente. Isto permite um fluxo maior de metabólitos e precursores para o epitélio secretor aumentando assim, a síntese de leite (Hanwell & Linzell, 1973b). Além disso, há evidências de que a liberação de hormônios pela hipófise anterior, principalmente a prolactina e o hormônio do crescimento, podem

ser os responsáveis pelo aumento do débito cardíaco nas ratas lactantes (Hanwell & Linzell, 1972). Além do aumento no débito cardíaco, as lactantes apresentam aumento do volume plasmático com consequente diminuição da concentração das proteínas plasmáticas totais (Hanwell & Linzell, 1973a; Suzuki *et al.*, 1993). As alterações que ocorrem durante o período lactacional podem influenciar a distribuição e a eliminação de compostos químicos neste período (Hallén *et al.*, 1996; Houpert *et al*., 1997, Sundberg *et al.*, 1998). Já foi demonstrado que durante a lactação a meia-vida biológica do mercúrio diminui cerca de 40%, tanto em animais quanto em humanos expostos ao metilmercúrio (Greenwood *et al*., 1978) e cerca de 50% nos animais expostos ao mercúrio inorgânico (Prester *et al*., 1994; 1997).

A prática do aleitamento materno vem sendo amplamente estimulada pelos profissionais e órgãos de saúde nos dias atuais. Baseado nisso, a administração de medicamentos e a exposição a agentes tóxicos ambientais durante este período requer especial atenção (Berlin & Briggs, 2005). O aleitamento é um meio de exposição dos lactentes aos agentes externos (medicamentos e agentes tóxicos ambientais) que a lactante está exposta (Anderson & Wolff, 2000; Dorea, 2004). Os compostos químicos passam do plasma para o leite materno principalmente por difusão, através das forças de equilíbrio criadas entre esses dois compartimentos (Needs & Brooks, 1985; Halbe, 1990). Na maioria das vezes, o nível plasmático dos agentes químicos é o fator determinante de sua presença no leite materno, porém a quantidade do agente difundido depende das seguintes características farmacológicas: (1) peso molecular; (2) lipossolubilidade; (3) capacidade de ligação às proteínas plasmáticas; (4) grau de ionização; (5) meia-vida de eliminação; (6) biodisponibilidade; e (7) concentração sanguínea materna. Desta forma, a excreção de xenobióticos do plasma para o leite é facilitada quando este apresenta: (1) baixo peso molecular, (2) elevada lipossolubilidade, (3) baixa capacidade de ligação às proteínas plasmáticas, (4) forma não ionizada, (5) elevada meia-vida de eliminação, (6) alta biodisponibilidade e (7) elevado poder de concentração no plasma materno (Needs & Brooks, 1985; Halbe, 1990; Ministério da Saúde, 2010). Outro aspecto importante é o pico sérico de concentração do xenobiótico. Usualmente, o pico na corrente sanguínea da mãe coincide com o pico de concentração no leite materno, sendo este último, menor (Ministério da Saúde, 2010). Estudos prévios realizados durante o período de lactação demonstraram que as formas inorgânicas de mercúrio são excretadas eficientemente no

leite materno (Sundberg *et al.*, 1991a; 1991b; 1998; Oskarsson *et al.*, 1996). Portanto, uma vez que a sensibilidade dos animais em desenvolvimento a diversos agentes tóxicos, incluindo os metais, pode diferir daquela observada em adultos (Jugo, 1976; Kostial *et al.*, 1978; Walsh, 1982; Webb & Holt, 1982; Pereira *et al.*, 1999), torna-se importante a realização de estudos que avaliem a toxicidade de formas inorgânicas de mercúrio no período neonatal.

2.4. Animais em desenvolvimento

Em roedores, o período neonatal tem seu início ao nascimento, sendo finalizado com o término da lactação (aproximadamente três semanas de idade) (Gottlieb *et al.*, 1977). Durante esse período, os roedores apresentam fases de desenvolvimento rápido pós-natal, subdivididas com base no desenvolvimento dos órgãos:

- primeira fase: inicia-se no dia do nascimento e estende-se até o 6º dia de vida;
- segunda fase: compreendida entre o 8º e o 13º dia de vida;
- terceira fase: ocorre do 17º ao 23º dia de vida.

Durante esses intervalos de desenvolvimento rápido há um aumento mais pronunciado do peso da maioria dos órgãos quando comparado com as taxas de desenvolvimento que se verificam imediatamente antes e depois desses intervalos (Kobayashi, 1963; Winick & Noble, 1965; Gottlieb *et al.*, 1977). Esse marcado crescimento e desenvolvimento dos órgãos é atribuído à maior quantidade de proteína, DNA e ácido ribonucléico (RNA) presentes nos órgãos em virtude da intensa síntese dos mesmos (Winick & Noble, 1965; Gottlieb *et al.*, 1977; Morgane *et al.*, 2002).

Segundo Smart & Dobbing (1971), neste período, classificado como o "período crítico" do desenvolvimento, os organismos parecem estar particularmente sensíveis aos efeitos da exposição a agentes externos. Diversos trabalhos demonstraram que animais em desenvolvimento são altamente sensíveis a insultos externos (Barone *et al.*, 2000; Rice & Barone, 2000; Mendola *et al.*, 2002; Morgane *et al.*, 2002). Esses insultos tanto podem ser sutis, como estresse, manipulação, subnutrição e privação do contato materno (Cowley & Widdowson, 1965; Goldman, 1965; Adlard & Dobbing, 1971; Dobbing & Sands, 1971; Franková & Blatníková, 1979; Rocha & Vendite, 1990), quanto podem ser graves, como exposições a metais pesados, praguicidas ou outros

agentes tóxicos (Ribeiro-Da-Silva *et al.*, 1994; Aston *et al.*, 1996; Moser, 2000; Ferri *et al.*, 2003; Roza *et al.*, 2005; Singh & Rishi, 2005; Dorea & Donangelo, 2006). Alguns trabalhos têm relatado uma alta sensibilidade dos animais expostos ao mercúrio durante o período neonatal (Rocha *et al*., 1993; 1995; Peixoto *et al*., 2003; 2004; 2007b; Peixoto & Pereira, 2007; Franciscato *et al*., 2009; 2011), sendo que foi demonstrado que esta sensibilidade é dependente do estágio de desenvolvimento pós-natal (Peixoto *et al.*, 2003; 2004). A grande vulnerabilidade desses organismos quanto a agentes químicos está relacionada à imaturidade dos órgãos e membranas e à incapacidade de metabolizar adequadamente os mesmos (Nies & Spielberg, 1996; Hodgson, 1997; Tyl, 1998).

2.5. Enzima d-aminolevulinato desidratase (d-ALA-D)

A d-ALA-D (E.C. 4.2.1.24), também denominada PBG-sintase, é uma enzima citosólica que catalisa a condensação de duas moléculas do substrato ácido d-aminolevulínico (d-ALA) para a síntese de uma molécula de porfobilinogênio (PBG) com a liberação de duas moléculas de água (Bernard & Lauwerys, 1987; Jaffe, 1995). A reação catalisada pela d-ALA-D, demonstrada na figura a seguir, faz parte da rota de biossíntese de compostos tetrapirrólicos, tais como o heme, clorofilas, corrinas e bilinas (Castelfranco *et al*., 1983). Estes desempenham papéis metabólicos importantes, principalmente como grupo prostético de proteínas.

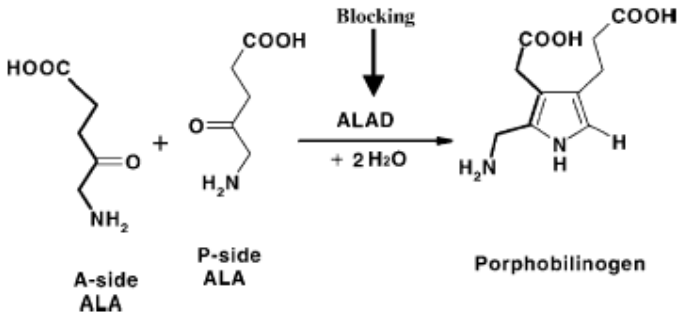

Fig 1. Síntese de porfobilinogênio (adaptado de Lee *et al.*, 2003).

A enzima d-ALA-D está amplamente distribuída nos organismos vivos, sendo seus maiores níveis encontrados em tecidos como medula óssea, fígado, rins e eritrócitos (Jaffe, 1995; Dessypris, 1998). O alto conteúdo de grupamentos sulfidrílicos dessa enzima (Shemin, 1976; Tsukamoto *et al.*, 1979) determina sua grande afinidade

por metais, preponderantemente, os divalentes (Border *et al.*, 1976; Scheuhammer, 1987). A ligação dos metais a esses grupamentos químicos altera sua capacidade catalítica (Border *et al.*, 1976; Despaux *et al.*, 1977; Davis & Avram, 1980; Scheuhammer, 1987).

Dados da literatura relatam que alguns metais, como o zinco, são ativadores da enzima d-ALA-D (Despaux *et al.*, 1977; Thompson *et al*., 1977; Nelson, 1981; Bernard & Lauwerys, 1987), enquanto que outros, como o mercúrio, o chumbo, o cádmio e o cobre, são inibidores da sua atividade (Thompson *et al*., 1977; Tsukamoto *et al.*, 1980; Nelson, 1981; Goering & Fowler, 1987; Goering, 1993; Rocha *et al*., 1993; 1995; 2001; Peixoto *et al*., 2003; 2004; 2007b; Franciscato *et al*., 2011). Além disso, alguns metais, como o alumínio e o estanho, foram classificados como ativadores da enzima d-ALA-D em baixas concentrações e inibidores em altas concentrações (Despaux *et al.*, 1977; Bernard & Lauwerys, 1987). As características de ubiquidade (Gibson *et al.*, 1955; Bernard & Lauwerys, 1987; Jaffe, 1995) e sua natureza sulfidrílica (Shemin, 1976; Tsukamoto *et al.*, 1979) são as responsáveis pela sua utilização como biomarcador da exposição a metais pesados. A inibição da atividade da d-ALA-D de eritrócitos humanos, por exemplo, é bastante utilizada como índice de exposição ao chumbo (Goering, 1993). Além disso, já foi demonstrado que a inibição da atividade desta enzima também reflete a intoxicação por mercúrio inorgânico tanto em ratos jovens (Rocha *et al.*, 1995; Peixoto *et al.*, 2003; 2004; 2007b; Roza *et al.*, 2005; Franciscato *et al.*, 2011) quanto em adultos (Perotoni *et al*., 2004a; 2004b; Emanuelli *et al.*, 1996; Augusti *et al.*, 2007; 2008).

3. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de manuscritos científicos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios manuscritos.

3.1. Manuscrito I

Difference in sensitivity to renal toxicity induced by mercuric chloride in lactating and non-lactating rats: the preventive role of zinc chloride

Alexandre M. Favero, Carina Franciscato, Cláudia S. Oliveira, Juliana S. F. Pereira, Claudia M. Bertoncheli, Sônia C. A. da Luz, Valderi L. Dressler, Érico M. M. Flores e Maria E. Pereira

Submetido ao Periódico *Food and Chemical Toxicology*

Elsevier Editorial System(tm) for Food and Chemical Toxicology
Manuscript Draft

Manuscript Number: FCT-D-09-01159R1

Title: Difference in sensitivity to renal toxicity induced by mercuric chloride in lactating and non-lactating rats: the preventive role of zinc chloride

Article Type: Full Length Article

Keywords: mercuric chloride; zinc chloride; nephrotoxicity; kidney; lactation and rats

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Abstract: This study evaluated the effects of $HgCl_2$ on renal parameters in non-lactating and lactating rats and theirs pups, and tested the preventive role of Zn. Rats received 27 mg/kg $ZnCl_2$ (or saline) for five consecutive days and 5 mg/kg $HgCl_2$ (or saline) for five subsequent days (s.c.). Decreased food intake, body weight, Zn levels and δ-aminolevulinic acid dehydratase (δ-ALA-D) activity in blood; and increased renal weight and blood Hg levels were observed in lactating and non-lactating rats from Sal-Hg and Zn-Hg groups. $ZnCl_2$ prevented the increase of Hg (partially) and the decrease of Zn (totally) levels in $HgCl_2$-non-lactating rats. Renal Hg levels were increased in all $HgCl_2$ groups and the $ZnCl_2$-exposure potentiates this effect in lactating rats. Non-lactating rats exposed $HgCl_2$ presented plasma urea and creatinine levels increased, δ-ALA-D activity inhibition and histopathological alterations in kidneys. $ZnCl_2$-exposure prevented several of these alterations. Pups Hg-exposed showed lower body and renal weight and an increase in the renal Hg levels. In conclusion, mercury-induced nephrotoxicity differs considerably between lactating and non-lactating rats. Moreover, the preventive effect of $ZnCl_2$ on renal toxicity induced by $HgCl_2$ in non-lactating rats suggests effectively that it serves as a promising alternative in the treatment of mercury poisoning cases.

Difference in sensitivity to renal toxicity induced by mercuric chloride in lactating and non-lactating rats: the preventive role of zinc chloride

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Running title: Difference in $HgCl_2$-induced renal toxicity

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Abstract

This study evaluated the effects of $HgCl_2$ on renal parameters in lactating and non-lactating rats, and tested the potential preventive role of Zn and the effects of metals on pups exposed to it through maternal milk. Lactating and non-lactating rats received 27 mg/kg $ZnCl_2$ (or saline) for five consecutive days and 5 mg/kg $HgCl_2$ (or saline) for five subsequent days (s.c.). The exposure of lactating rats to metals began on lactation day 3. Decreased food intake, loss of body weight, and increased renal weight were observed in lactating and non-lactating rats from Sal–Hg and Zn–Hg groups. A significant increase in Hg and decrease in Zn levels in blood of lactating and non-lactating rats exposed to $HgCl_2$ were observed. Pre-exposure to $ZnCl_2$ prevented the increase of Hg (partially) and the decrease of Zn (totally) levels induced by $HgCl_2$ only in non-lactating rats. Renal Hg levels were increased in all $HgCl_2$ groups and the $ZnCl_2$-exposure potentiates this effect in lactating rats. Plasma urea and creatinine levels were increased and renal d-aminolevulinic acid dehydratase (d-ALA-D) activity was inhibited only in non-lactating rats exposed to $HgCl_2$ and the $ZnCl_2$-exposure prevented these alterations. Blood d-ALA-D activity was inhibited in both lactating and non-lactating rats exposed to $HgCl_2$ and pre-exposure to $ZnCl_2$ partially prevented this inhibition in non-lactating rats. $HgCl_2$-exposure caused histopathological alterations in kidneys of non-lactating rats. Hg-exposed pups showed lower body weight gain and renal weight and an increase in the renal Hg levels. Thus, we demonstrated that mercury-induced nephrotoxicity differs considerably between lactating and non-lactating rats. Moreover, the preventive effect of $ZnCl_2$ on renal toxicity induced by $HgCl_2$ in non-lactating rats suggests that it serves as a promising alternative in the preventive treatment of mercury poisoning cases.

Keywords: mercuric chloride, zinc chloride, nephrotoxicity, kidney, lactation and rats

1. Introduction

Mercury is present in different forms in the environment (elemental, organic and inorganic) and has been recognized as a hazardous environmental pollutant. Despite this, it is widely used in certain types of batteries (Clarkson, 1997) and is very important as constituent of energy saving light bulbs which are compulsory now in Europe (Commission Regulation (EC), 2009) and other regions.

Exposure to elemental and organic mercury primarily affects the central nervous system (Harada, 1978; Bornhausen et al., 1980; Sarafian and Verity, 1991; Fredriksson et al., 1992; Sakamoto et al., 1993; Goyer, 1995) while inorganic mercury has a non-uniform distribution after absorption, being accumulated mainly in kidneys causing renal injury (Magos, 1974; Zalups, 1993; Goyer, 1995; Emanuelli et al., 1996; Tanaka-Kagawa et al., 1998; WHO, 2003). The primary function of the kidney is the excretion of waste products. The kidney also plays a significant role in regulation of body homeostasis, regulating extracellular fluid volume and electrolyte balance. Thus, toxicity to the kidney could affect any of these functions. Generally, increases in plasma urea and creatinine levels are reflective of excretory function damage and are used clinically to diagnose kidney damage (Hodgson and Levi, 2004).

During lactation several physiological conditions are altered, such as increase in blood and plasma volume, decrease in total plasma protein, and increases in cardiac output and blood flow through organs as a response to increasing demands from the young during this period (Hanwell and Linzell, 1973; Suzuki et al., 1993). These changes may certainly influence the distribution and elimination of chemical compounds (Palminger et al., 1996; Houpert et al., 1997, Sundberg et al., 1998). In fact, the Hg biological half-time decreases by 40% both in humans and animals exposed to methyl mercury during lactation (Greenwood et al., 1978) and by 50% in animals exposed to inorganic mercury (Prester et al., 1994; 1997). One study with female Wistar rats has demonstrated that lactation delayed the onset of weight loss, shortened the time between the end of treatment and the onset of weight gain, accelerated the elimination of mercury from the whole body and prevented the development of severe co-ordination disorders after methyl mercury intoxication (Magos et al., 1980). Although organ distribution and toxicity of inorganic mercury have been studied by numerous

investigators, differences in susceptibility to the renal toxicity of inorganic mercury related to the lactation period are still scarce in the literature.

The sensitivity of developing animals to various compounds (including metals) may differ from that observed in adults (Jugo, 1976; Kostial et al., 1978; Walsh, 1982; Webb and Holt, 1982; Pereira et al., 1999), making the study of the metal effects on developing organisms important. In fact, young animals, mainly during the first days after birth, seem to be more sensitive than adults to toxic agents (Null et al., 1973; Jugo, 1976; Nielsen and Andersen, 1996). This vulnerability may be related to organ immaturity (Schulz et al., 1962; Winick and Noble, 1965). Recently, it was reported that exposure to mercury chloride ($HgCl_2$), a form of inorganic mercury, for five consecutive days alters renal function (Peixoto and Pereira, 2007), kidney weight (Rocha et al., 1995; Peixoto et al., 2003; Roza et al., 2005) as well as inhibits d-aminolevulinic acid dehydratase (d-ALA-D) activity from kidneys of young rats (Rocha et al., 1995; Peixoto et al., 2003; Roza et al., 2005). In fact, d-ALA-D is a sulfhydryl-containing enzyme and has been assumed to be a target for heavy metals exposure, including mercury (Peixoto et. al., 2004). The pre-exposure to zinc, an essential trace element, prevents or alleviates most of the toxic effects caused by mercury (Peixoto et al., 2003). This effect of zinc is of interest, since metal intoxication is usually treated with chelating agents, which are not specific and are often more toxic than the metal itself (Bapu et al., 1994; Roza et al., 2005).

In this context, the aim of this study was to evaluate the effects of $HgCl_2$ exposure on renal parameters indicative of nephrotoxicity in lactating and non-lactating rats. In addition, the present work provides data on the preventive role of $ZnCl_2$ on the nephrotoxicity induced by mercury and the effects of metals on pups exposed to it exclusively via maternal milk.

2. Materials and methods

2.1. Chemicals

Mercuric chloride, zinc chloride, sodium chloride, potassium phosphate monobasic and dibasic, absolute ethanol, ether, sodium hydroxide, trichloroacetic acid, nitric acid, sulfuric acid, o-phosphoric acid, perchloric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). d-Aminolevulinic acid (d-ALA), bovine serum albumin and Coomassie brilliant blue G were obtained from Sigma (St. Louis,

MO, USA); ρ-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han., Germany). The kits for determination of creatinine and urea were acquired from LABTEST (Lagoa Santa/ MG/ Brazil).

2.2. Animals

Adult female *Wistar* rats (pregnant and non-pregnant), 90 days old, obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12-h light/dark cycle and at a controlled temperature (22 ± 2ºC). Pregnant rats were allowed to deliver and wean their pups until lactational day (LD) 13. Animals had free access to water and commercial food (GUABI, RS, Brazil) and were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Exposure to metals

Lactating and non-lactating rats were distributed on a random basis into four exposure groups and housed in individual standard polypropylene plastic cages (41 x 34 x 18 cm). Animals were subcutaneously (s.c.) exposed to 0.9% NaCl (saline solution) or metals dissolved in saline solution at a volume of 1 mL/kg body weight (b.w.) following the experimental design:

I - Exposure protocol of lactating rats

Group 1 (Sal–Sal): lactating rats received saline for ten consecutive days (LD 3 to 12) to serve as control.

Group 2 (Sal–Hg): lactating rats received saline for five consecutive days (LD 3 to 7). On five subsequent days (LD 8 to 12) animals were daily exposed to 5 mg/kg $HgCl_2$.

Group 3 (Zn–Sal): lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals received saline.

Group 4 (Zn–Hg): lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals were daily exposed to 5 mg/kg $HgCl_2$.

The suckling pups were exposed to metals exclusively through maternal milk. It is important to mention that the transport of inorganic mercury into milk was previously demonstrated in the literature (Sundberg et al., 1991; Yoshida et al., 1994).

II - Exposure protocol of non-lactating rats

Group 1 (Sal–Sal): non-lactating rats received saline for ten consecutive days (days 1 to 10) to serve as control.

Group 2 (Sal–Hg): non-lactating rats received saline for five consecutive days (days 1 to 5). On five subsequent days (days 6 to 10) the animals were daily exposed to 5 mg/kg $HgCl_2$.

Group 3 (Zn–Sal): non-lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals received saline.

Group 4 (Zn–Hg): non-lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals were daily exposed to 5 mg/kg $HgCl_2$.

Animals were weighed immediately before exposure to metals to adjust the dose. The doses, exposure route and time period which lactating rats were exposed to metals (LD 3 to 12) were chosen according to previous studies performed by our research group, who showed that 5 mg/kg/day $HgCl_2$ caused toxic effects that were prevented by 27 mg/kg/day $ZnCl_2$ in suckling rats directly exposed to metals and killed 24h after the end of mercury exposure (Peixoto et al. 2003; 2007; 2008; Peixoto and Pereira, 2007; Franciscato et al., 2009).

All animals were daily observed throughout the study for mortality and signs of toxicity. Water and food consumption of lactating and non-lactating rats were monitored daily during the period of exposure to metals.

2.5. Tissue preparation

Twenty-four hours after the last administration of $HgCl_2$ lactating (and their pups) and non-lactating rats were weighed, sedated with ether and euthanized by decapitation. Blood samples were collected in tubes with heparin and centrifuged at 2000×*g* for 10 min at 4°C to obtain the plasma. For d-ALA-D activity assay, blood was hemolyzed in distilled water 1:4 (v/v) with agitation in ice bath for 10 min. Kidneys were removed and weighed. For d-ALA-D activity assay, kidneys were quickly placed on ice and homogenized in 5 volumes of NaCl (150 mM, pH 7.4) with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 8000×*g* for 30 min at 4°C and the supernatant fraction was used in the enzyme assay. For determination of metal (Zn and Hg) levels, tissue samples were placed into vials

and then frozen at -20 ºC until the day of analysis. For histological examination, kidneys were quickly removed, decapsulated and divided longitudinally into two equally sized pieces. One piece was fixed in 10% neutral-buffered formalin for 48 h, routinely processed, embedded in paraffin and sectioned. The sections were cut 3µm-thick for histopathological examination.

2.6. Biochemical determinations

2.6.1. Urea

The incubation, at 37ºC for 5 min, was started by adding 10 µL of plasma sample to a medium containing phosphate buffer 19.34 mmol/L pH 6.9, sodium salicylate 58.84 mmol/L, sodium nitroprusside 3.17 mmol/L, and urease (=12.63 UK/L). The reaction was stopped by adding the oxidant solution (final concentrations: NaOH 0.07 mol/L and sodium hypochlorite 3.01 mmol/L) and the mixture was incubated for 5 min to achieve color development. The absorbance of indophenol blue formed was measured at 600 nm. Urea concentration was read from an indophenol calibration curve.

2.6.2. Creatinine

The estimation of plasma creatinine was carried out by measuring the quantity of product formed, creatinine picrate, and by utilizing creatinine as standard. The reaction was conduced in a medium containing picric acid 20.2 mmol/L and NaOH 145.4 mmol/L in a thermostatized cuvette at 37ºC with 100 µL of plasma added. The absorbance was recorded at 510 nm.

2.6.3. d-ALA-D activity

The enzymatic activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen - PBG) formation, except that 76 mmol/L sodium phosphate buffer (pH 6.8) and 2.2 mmol/L d-ALA were used. The incubation was initiated by adding 200 µL of 8000×*g* supernatant of the kidney homogenate or 200 µL of hemolyzed blood and was carried out for 90 min (kidney) and 120 min (blood) at 39 ºC. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 mol/L and the PBG was measured with Ehrlich's reagent at 555 nm, using the molar absorption coefficient of 6.1×10^4 for Ehrlich-PBG salt. The

specific enzymatic activity was expressed as nmol of PBG formed per hour per mg protein.

2.6.4. Protein determination

Protein concentrations were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.7. Analysis of the metals

2.7.1. Digestion procedure

Samples weighing around 250 mg each were transferred to quartz vessels. Concentrated HNO_3 (6 mL) was added to each vessel, which was capped and placed into the microwave oven. Samples were digested using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280°C, Anton Paar, Graz, Austria). The microwave oven operated at 1400 W for 30 min and at 0 W for 20 min. The last step was used for cooling. After digestion, samples were diluted with water to 25 mL and transferred to graduated polypropylene vials. Colorless and clear solutions were obtained after the digestion step. Spike recovery tests and biological certified reference material (SRM NIST 1577, bovine liver) were carried out to validate the results. Blanks were run and analyzed after each ten measurements in order to check possible memory effects for all elements.

2.7.2. Quantification of metals

Metal analyses were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes. A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for Hg determinations. A deuterium background corrector was used for all the determinations. Hollow cathode lamps were used and operated at 4 mA for both metals. Wavelength was set at 213.9 and 253.7nm, for Zn and Hg, respectively, and the spectral band pass at 0.5 nm for both metals. Integrated absorbance (peak area) was used for all measurements. Heating program for Zn was carried out according to the recommendations of the manufacturer. For Hg determination, 3 mol/L HCl and 0.25% m/v $NaBH_4$ solutions were used as acid medium and reductant, respectively. Argon was

used as purge gas. Results for all elements determined were periodically evaluated by measurements of analytical standards (each 10 measurements) and also by the digest analysis of certified reference material SRM NIST 1577 (each 3 h of measurements). If the result for standard checking presented a bias higher than 5% a recalibration, the procedure was performed.

2.8. Histopatological examination

2.8.1. Histological and Histochemical Methods

For routine histology sections were stained with Haematoxylin and Eosin (H&E), Periodic-acid Schiff (PAS) and Picrosirius and were examined with light microscope.

The H&E stain was performed on kidney to identify tubulointerstitiais changes, necrosis and inflammation. A picrosirius red stain was performed on kidney sections to identify matrix deposition within the interstitium. In brief, 3µm-thick sections were deparaffinized, rehydrated and then stained with 0.1% Sirius red (Polysciences, Inc., Warrington, PA) in saturated picric acid (picrosirius red). After 60 min the slide were washed in water and strongly stained for haematoxylin for 10 min and rapidly dehydrated and mounted in balsam. The PAS reaction stains carbohydrates and carbohydrates-rich macromolecules. It is used to visualize the basal membrane that underlies epithelia and atrophic tubules containning PAS-positive hyaline cast. The samples were cutted in 3 µm-thick sections were deparaffinized, rehydrated, followed of addition of periodic acid 1% for 20 min. The slides were washed in water and additioned Schiff solution. After 30 min the slides were washed in water and stained with haematoxylin for 1 min and dehydrated and mounted in balsam. The histological and histochemical preparations were examined with a light microscope (Bioval L2000C). Digital images were captured (Biocam VI-6633/ color CCD camera) separately for each stain and group and the images were merged with the software accompanying the camera (Honestech AVR2,5).

2.9. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test when appropriate. Comparisons among all group treatment means were made. Different letters were used to indicate significant differences among

groups. Lactating and non-lactating rats were independently analyzed. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Effects of exposure to metals in lactating and non-lactating rats

3.1.1. Mortality rate

No mortality was observed in any group of lactating rats. Non-lactating rats exposed to $HgCl_2$ (Sal–Hg) showed high mortality rate (~32%) and those pre-exposed to $ZnCl_2$ (Zn–Hg) showed only 5% mortality rate (Fig. 1). No mortality was observed in control and $ZnCl_2$ groups of non-lactating rats.

3.1.2. Body and renal weights

Body and renal weights are illustrated in Figs. 2 and 3, respectively. One-way ANOVA revealed that body weight gain over 5 days was significantly affected by metal administrations in both lactating [$F(3,23)= 21.35$, $P<0.001$] and non-lactating [$F(3,22)= 27.41$, $P<0.001$] rats. This effect was due to the significant body weight loss of lactating (Fig. 2a) and non-lactating (Fig. 2b) rats exposed to $HgCl_2$ when compared to their respective control groups ($P<0.05$, Duncan's multiple range test). Previous exposure to $ZnCl_2$ did not prevent the effects of $HgCl_2$ on this parameter.

Regarding renal weight, one-way ANOVA revealed significant alteration by metal administrations in both lactating [$F(3,23)= 18.39$, $P<0.001$] and non-lactating [$F(3,22)= 22.75$, $P<0.001$] rats. Lactating and non-lactating rats exposed to $HgCl_2$ presented an increase of approximately 55% and 95% in renal relative weight, respectively, when compared to their respective control group ($P<0.05$, Duncan's multiple range test) (Fig. 3). Previous exposure to $ZnCl_2$ partially prevented the increase induced by exposure to $HgCl_2$ in lactating ($P<0.05$, Duncan's multiple range test), but it was unable to prevent this increase in non-lactating rats (Fig. 3).

3.1.3. Food and water intake

The effect of metal administrations on food intake is illustrated in Fig. 4. One-way ANOVA revealed a significant effect of metal administrations on food intake in both lactating [$F(3,23)= 21.21$, $P<0.001$] and non-lactating [$F(3,22)= 21.61$, $P<0.001$] rats. This effect was due to a significant lower food intake by lactating (about 45%)

(Fig. 4a) and non-lactating (about 70%) (Fig. 4b) rats exposed to $HgCl_2$ when compared to their respective control groups ($P<0.05$, Duncan's multiple range test). Previous exposure to $ZnCl_2$ did not prevent the effects of $HgCl_2$ on this parameter.

Water intake was altered neither in lactating nor non-lactating rats exposed to metals (data not shown).

3.1.4. Urea and creatinine levels

Effects of metal administrations on renal function were assessed by urea and creatinine levels and are illustrated in Figs. 5 and 6, respectively. Plasma urea and creatinine levels were not altered by metal administrations in lactating dams. On the other hand, one-way ANOVA revealed that both parameters were significantly altered by metal administrations in non-lactating rats [urea, $F(3,22)= 9.24$, $P<0.001$ and creatinine, $F(3,22)= 3.10$, $P<0.05$]. Non-lactating rats exposed to $HgCl_2$ presented remarkable increase in urea (up to three times) and creatinine (up to four times) levels when compared to the other groups. Previous exposure to $ZnCl_2$ was able to prevent these increases ($P<0.05$, Duncan's multiple range test).

3.1.5. Renal d-ALA-D activity

The effect of metal administrations on renal d-ALA-D activity is illustrated in Fig. 7. The enzyme activity was not altered by metal administrations in lactating dams. Conversely, the one-way ANOVA revealed that it was significantly altered by metal administrations in non-lactating rats [$F(3,22)= 6.50$, $P<0.005$]. When exposed to $HgCl_2$, these animal presented a decrease in d-ALA-D activity (about 23%) compared to the control group. $ZnCl_2$ pre-exposure prevented this inhibitory effect induced by $HgCl_2$ ($P<0.05$, Duncan's multiple range test).

3.1.6. Blood d-ALA-D activity

One-way ANOVA revealed significant effects of metal administrations on blood d-ALA-D activity in lactating rats [$F(3,8)= 12.48$, $P<0.05$] and in non-lactating rats [$F(3,12)= 5.20$, $P<0.05$]. The animals exposed to $HgCl_2$ presented a significant inhibition in the enzyme activity (about 67% and 83% in lactating and non-lactating rats, respectively) when compared to the control group ($P<0.05$, Duncan's multiple range test). Previous exposure to $ZnCl_2$ did not prevent the alteration induced by

mercury on blood d-ALA-D activity in lactating but was able in partially to prevent the inhibition on blood d-ALA-D activity induced by exposure to $HgCl_2$ (Table 1).

3.1.7. Blood Hg and Zn levels

One-way ANOVA revealed significant effects of metal administrations on Hg [$F(3,10)= 77.42$, $P<0.001$] and Zn [$F(3,10)= 7.40$, $P<0.01$] levels in blood of lactating rats. Both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented a significant increase in blood Hg levels and a significant decrease in blood Zn levels when compared to other groups ($P<0.05$, Duncan's multiple range test). Previous exposure to $ZnCl_2$ did not prevent the alteration induced by mercury on blood metal (Hg and Zn) levels (Fig. 8a).

Regarding non-lactating rats, one-way ANOVA showed significant effects of metal administrations on Hg [$F(3,8)= 6.20$, $P<0.05$] and Zn [$F(3,8)= 9.13$, $P<0.01$] levels in blood. A significant increase in Hg and a decrease in Zn levels were observed in blood of non-lactating rats exposed to $HgCl_2$ when compared to the control group ($P<0.05$, Duncan's multiple range test). Pre-exposure to $ZnCl_2$ partially prevented the increase of Hg and totally prevented the decrease of Zn levels induced by exposure to $HgCl_2$ (Fig. 8a).

3.1.8. Kidney Hg and Zn levels

One-way ANOVA revealed significant effects of metal administrations on Hg [$F(3,10)= 148.93$, $P<0.001$] and Zn [$F(3,10)= 6.01$, $P<0.05$] levels in kidneys of lactating rats. Lactating rats exposed to $HgCl_2$ showed a significant increase in renal Hg levels and the previous exposure to $ZnCl_2$ potentiates this effect. Lactating rats of both $ZnCl_2$-exposed groups presented higher renal Zn levels than the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 8b).

In relation to non-lactating rats, one-way ANOVA revealed significant effects of metal administrations on Hg [$F(3,8)= 99.93$, $P<0.001$] and Zn [$F(3,8)= 21.57$, $P<0.001$] levels in kidneys. Animals from both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented a significant and similar increment in renal Hg levels when compared to the other groups ($P<0.05$, Duncan's multiple range test). Non-lactating rats of both groups exposed to $ZnCl_2$ showed a significant increase in renal Zn levels when compared to the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 8b).

3.1.9. Histopathological examination

No histopathological alterations were observed in kidneys of lactating rats from Sal–Sal, Zn–Sal and Zn–Hg groups. Only focal changes with some tubules distended and rare atrophic tubules showing flattened ephitelium and proteinaceous filling the lumen were observed in the Sal–Hg group (data not shown).

Both groups of non-lactating rats exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented remarkable hitopathological lesions in the kidneys such as renal tubules atrophy with tubular dilatation, formation of protein casts and rarely mononuclear cell infiltration. Thyroid-type atrophic tubules have flattened epithelium and PAS-positive proteinaceous filling the lumen. The Picrosirius stain revealed mild degree of interstitial collagen deposition. No histopathological alterations were observed in kidneys of non-lactating rats from Sal–Sal and Zn–Sal groups (Fig 9).

3.2. Effects of exposure to metals in suckling pups

3.2.1. Mortality rate

No mortality was observed in any group of suckling pups (data not shown).

3.2.2. Body and renal weights

One-way ANOVA revealed that body weight of suckling pups was significantly affected by metal administrations [$F(3,23)= 42.39$, $P<0.001$]. Pups from Sal–Hg and Zn–Hg groups had a lower body weight gain during the period of exposure to $HgCl_2$ (LD 8 to 12) than the other groups ($P<0.05$, Duncan's multiple range test). At LD 13, Hg–exposed pups presented body weight significantly lower when compared to the control group ($P<0.05$, Duncan's multiple range test). The body weight gain of suckling pups was not altered during the period of exposure to $ZnCl_2$ (LD 3 to 7) (Table 2).

One-way ANOVA revealed that absolute kidney weight was significantly altered by metal administrations [$F(3,23)= 14.18$, $P<0.001$] in suckling pups. Pups from Sal–Hg and Zn–Hg groups showed a decrease in the absolute renal weight of around 15% and 23%, respectively, when compared to the control group ($P<0.05$, Duncan's multiple range test). Relative kidney weights were similar among groups of suckling pups (Table 2).

3.2.3. Urea and creatinine levels

Plasma urea and creatinine levels were not altered by metal administrations in suckling pups (Table 3).

3.2.4. Renal d-ALA-D activity

Renal d-ALA-D activity was not altered by metal administrations in suckling pups (Table 3).

3.2.5. Kidney Hg and Zn levels

One-way ANOVA revealed significant effects of metal administrations [$F(3,11)= 10.16$, $P<0.005$] on Hg levels in kidneys of suckling pups. Pups from Sal–Hg and Zn–Hg groups presented a significant increase in renal Hg levels when compared to the other groups ($P<0.05$, Duncan's multiple range test). Renal Zn levels were similar among groups of suckling pups (Table 3).

3.2.6. Histopathological examination

No histopathological alterations were observed in any group of suckling pups (data not shown).

4. Discussion

Although numerous investigators have studied organ distribution and toxicity of inorganic mercury, differences in the susceptibility of adult animals to inorganic mercury damage related to the lactation period are still scarce in the literature. Data presented in this study demonstrate that mercury-induced nephrotoxicity differs considerably between lactating and non-lactating rats. Indeed, mercury exposure increases plasma urea and creatinine levels, inhibits renal δ-ALA-D activity and induces histopathological alterations only in non-lactating rats. Additionally, it was demonstrated that $ZnCl_2$ was able to prevent renal alterations induced by inorganic mercury in non-lactating rats, similarly to previous demonstrated in young rats (Peixoto et al., 2003; Peixoto and Pereira, 2007), effectively suggesting that it may serve as a promising preventive alternative treatment of mercury poisoning cases.

Loss of body weight was observed in the groups of lactating and non-lactating rats exposed to $HgCl_2$. Body weight changes were likely a result of the reduced food intake, which was observed in lactating and non-lactating rats exposed to $HgCl_2$. In fact, the anorexigenic effects of mercury have been well documented in the literature (Freundt and Ibrahim, 1990; Counter and Buchanan, 2004). It is also important to emphasize that lactating and non-lactating rats exposed to $HgCl_2$ had normal water ingestion (data not shown) and did not present visual signs of dehydration.

The blood d-ALA-D activity was inhibited both in lactating and in non-lactating rats exposed to $HgCl_2$, reflecting the blood Hg levels and confirming previous studies that showed this enzyme as a biomarker of exposure to mercury (Bernard and Lauwerys, 1987; Oskarsson and Fowler, 1987; Peixoto et al., 2004). It is important to note that blood d-ALA-D activity was inhibited at Hg concentrations lower than those found in kidneys. In agreement, Rocha et al. (1995) have demonstrated that blood d-ALA-D enzyme is more sensitive to mercury than the renal. Therefore, despite differences with respect to sensitivity to the effects of mercury on renal parameters in the lactation period, the blood d-ALA-D enzyme may be a good indicator of mercury exposure also for lactating rats.

$HgCl_2$ has been long recognized as a nephrotoxic agent (ATSDR, 1994; Zalups and Lash, 1994). The kidneys are the primary organs that accumulate inorganic mercury and the presence of this metal within the tubular cell may be seen within a few hours after exposure (Zalups, 1993). In the present study, lactating and non-lactating rats showed an increase in renal Hg levels after $HgCl_2$ exposure. The previous exposure to $ZnCl_2$ did not prevent the renal Hg accumulation in non-lactating and enhanced this effect in lactating rats. Nevertheless, the increase in renal mercury concentration caused by prior exposure to $ZnCl_2$ was unable to induce any alteration in renal parameters evaluated in lactating rats. The effect of $ZnCl_2$ on increasing the concentration of mercury in kidneys of animals pre-exposed to it, and then exposed to $HgCl_2$, was previously demonstrated by our research group (Peixoto et al., 2003; 2007; Franciscato et al., 2011). Biochemical parameters indicative of mercury-induced nephrotoxicity were severely altered only in non-lactating rats. Lactating and non-lactating rats exposed to $HgCl_2$ presented an increase of approximately 55% and 95% in renal weight, respectively. Although it has been reported that kidney weight increase is a relatively sensitive index of nephrotoxicity (Sharratt and Frazer, 1963; Kluwe et al., 1981;

Simmons, et al., 1995), some investigators demonstrated that only a severe renal injury, that results in 50–75% reduction in glomerular filtration rate, increases plasma urea and creatinine levels (Goldstein and Schnellmann, 1996; Dinour and Brezis, 1997). In our study, non-lactating rats (but not lactating) exposed to $HgCl_2$ presented an increase in renal weight followed by a pronounced increase in plasma urea and creatinine levels, which indicates that these animals experienced severe renal injury (Goldstein and Schnellmann, 1996; Dinour and Brezis, 1997). The histopathological evaluation corroborate these findings inasmuch as only non-lactating rats exposed to $HgCl_2$ presented kidney histopathological alterations, including interstitial fibrosis (characterized by increased deposition of interstitial collagen), tubular dilatation and flattening of epithelial cells with extensive proteinaceous tubular casts (an indicator of extensive tubular damage). Kidney fibrosis contributes to renal injury and correlates well with the overall loss of renal function (Cohen, 1995). It is important to mention that a high mortality rate was observed in the group of non-lactating rats exposed to $HgCl_2$. In conformity, Barnes et al. (1980) have demonstrated that Hg^{2+} ions are concentrated in the kidney after $HgCl_2$ exposure, producing damage to the renal tubular system, and ultimately leading to death from uremia.

The distinct responses of lactating and non-lactating rats observed in this study are propably associated with some of several physiological changes that occur during lactation (Hanwell and Linzell, 1973; Suzuki et al., 1993). These physiological changes, such as increase in blood and plasma volume, decrease in total plasma protein and increases in cardiac output and blood flow, could affect the pharmacokinetic of a variety of chemicals (Palminger et al., 1996; Houpert et al., 1997, Sundberg et al., 1998), including mercury. In fact, during lactation, the biological half-time of inorganic mercury decreases by 50% due to a higher rate of excretion (i.e., 3.5 days in lactating rats compared to ~7 days in non-lactating adult females) (Prester et al., 1994; 1997). One important mechanism that could explain, at least in part, the reduced toxicity of mercury in lactating rats is the increase in metallothionein (MT) synthesis during this period (Solaiman et al., 2001). MT are ubiquitous proteins characterized by a high affinity for metals (Chan et al., 2002; Dabrio et al., 2002). They have been implicated in the homeostatic control of metal metabolism and cellular adaptation to stress and may be modified by exposure to several stressors including metals, chemical compounds, physical stress, ionizing radiation, and hormones (Dunn et al., 1987; Shimada et al.,

1997). Indeed, it is well known that during mid-lactation an induction of MT occurs, suggesting to be involved in meeting the increased demands of essential metals in the neonate (Solaiman et al., 2001). In our study, lactating rats were exposed to $HgCl_2$ close to the period of lactation (mid-lactation) recognized as being the peak period of MT synthesis in rodents (Solaiman et al., 2001). Therefore, assuming the high cysteine content in the MT molecule, it is possible that this protein sequesters a high amout of this toxic metal in an inert complex, making it less available to interact with sensitive organelles or enzyme systems (Klaassen et al., 1999; Nath et al., 2000; Romero-Isart and Vasak, 2002; Peixoto et al., 2003). From a toxicological point of view, the protection of lactating rats from mercury toxicity is very important since the development of neonates is highly dependent on their mother's health during this period.

The previous exposure to Zn, an essential trace metal required for normal growth, development and functioning of animal species, prevented mercury-induced nephrotoxicity and the inhibition of renal d-ALA-D activity in non-lactating rats. One of the potential mechanisms of preventive effects of Zn on mercury-induced nephrotoxicity has been associated with its ability to induce the synthesis of detoxifying proteins, such as MT or other metal-binding proteins (Dunn et al., 1987; Zalups and Cherian, 1992; Kondoh et al., 2003; Peixoto et al., 2003, 2007; Szczurek, et al., 2009). In line with this, it was previously demonstrated that pre-exposure to $ZnCl_2$ prevents the renal damage and the inhibitory effect on the renal d-ALA-D activity induced by $HgCl_2$ in young rats concomitantly to the increase of MT levels (Peixoto et al., 2003; 2007). Several authors have demonstrated that kidney damage caused by inorganic mercury is prevented by pre-induction of renal MT because intracellular mercury in the kidney is firmly trapped by MT (Webb and Magos, 1976; Zalups and Cherian, 1992). Based on this, we hypothesized that an increase in renal MT levels induced by Zn may be the mechanism responsible, at least in part, for the reduction in mercury toxicity in non-lactating rats.

Since sensitivity of developing animals to various compounds (including metals) may differ from that observed in adults (Jugo, 1976; Kostial et al., 1978; Walsh, 1982; Webb and Holt, 1982; Pereira et al., 1999), we also evaluated the effects of metals on pups exposed to them via maternal milk. The transport of inorganic mercury into milk was previously demonstrated in rats by Sundberg et al. (1991) and in guinea pigs by

Yoshida et al. (1994). Indeed, Sundberg et al. (1991) demonstrated that mercury levels in milk are linearly correlated to the mercury levels in kidneys of suckling pups after exposure to mercury via milk. In the present study, an increase in kidney mercury levels of suckling pups was observed for both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg). Interestingly, our results showed that the increase of mercury levels observed in kidneys of pups seems to be insufficient to cause alterations on biochemical parameters related to renal damage. In fact, the mercury levels in kidneys of suckling pups exposed to mercury via milk is low, ranging from only 0.90 to 1.30% of the level in maternal kidneys. The only significant physiological change observed in pups was lower body weight gain during the period of exposure to this metal that can be consequence of dam weight decrease due to low food ingestion. In contrast, when young rats were directly exposed to $HgCl_2$ (at the same dose and developmental period) an increase in kidney Hg levels (Peixoto et al., 2003; 2007) followed by alterations in renal functions (Peixoto and Pereira, 2007) and inhibition of renal d-ALA-D activity (Rocha et al., 1995; Peixoto et al., 2003) was demonstrated.

In conclusion, renal toxicity induced by inorganic mercury differs considerably between lactating and non-lactating rats. Certainly, this could be related to some of the several physiological changes that occur during the lactation period. Additional studies should be performed to further examine the differential sensibility of lactating and non-lactating rats against $HgCl_2$ toxicity. Moreover, the preventive effect of $ZnCl_2$ on renal alterations induced by mercury in non-lactating rats effectively suggests that it may serve as a promising preventive alternative treatment of mercury poisoning cases.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements:

Financial support-provided by FINEP research grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net)’’ #01.06.0842-00. M.E.P. (307814/2008-4) and C.S.O. are recipients of CNPq fellowships; A.M.F. and C.F. were recipients of CNPq (142297/2006-2) and CAPES fellowships, respectively.

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Legends

Figure 1. Survival rate (%) in lactating and non-lactating rats during the period of exposure to $HgCl_2$. Animals were exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days.

Figure 2. Body weight gain (g) of lactating (a) and non-lactating (b) rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different ($P<0.05$, Duncan's multiple range test).

Figure 3. Renal weight (relative-to-body weight) of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different in the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 4. Food intake (g/ day/ 100 g b.w.) of lactating (a) and non-lactating (b) rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different ($P<0.05$, Duncan's multiple range test).

Figure 5. Plasma urea levels (mg/ dL) of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different in the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 6. Plasma creatinine levels (mg/ dL) of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7).

Bars labeled with different letters are significantly different in the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 7. Renal d-ALA-D activity of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different in the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 8. Mercury and zinc levels in blood (a) and kidneys (b) of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 3–5). Bars labeled with different letters are significantly different in the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 9. Histopathological evaluation in kidneys of non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days.

Plate 1. Kidney sections stained by haematoxylin and eosin (H&E 20×): Sal–Sal (A) and Zn–Sal (C) groups showed morphologically normal glomerulli and renal tubules. Sal–Hg (B) and Zn–Hg (D) groups showed remarkable hitopathological lesions such as renal tubules atrophy with tubular dilatation (arrow 1) and rarely mononuclear cell infiltration (arrow 2).

Plate 2. Kidney sections stained by Periodic-acid Schiff (PAS 20×): Sal–Sal (A) and Zn–Sal (C) groups showed absence of tubular protein casts. Sal–Hg (B) and Zn–Hg (D) groups showed thyroid-type atrophic tubules with flattened epithelium (arrow 1) and PAS-positive proteinaceous filling the lumen (star).

Plate 3. Kidney sections stained by Picrosirius red (Picrosirius 20×)**:** Sal–Sal (A) and Zn–Sal (C) groups showed absence of collagen deposition. Sal–Hg (B) and Zn–Hg (D) groups showed mild degree of interstitial collagen deposition (arrow).

Tables

Table 1. Blood d-ALA-D activity (nmol PBG/ mg protein/ h) in lactating and non-lactating rats exposed to $ZnCl_2$ and $HgCl_2$

Groups	Sal–Sal	Sal–Hg	Zn–Sal	Zn–Hg
Lactating	0.525 ± 0.015^{a}	0.175 ± 0.055^{b}	0.450 ± 0.070^{a}	0.265 ± 0.015^{b}
Non-lactating	0.310 ± 0.072^{ab}	0.053 ± 0.028^{c}	0.465 ± 0.108^{a}	0.165 ± 0.082^{bc}

Data are expressed as mean ± S.E.M. (n = 3–4) and the values followed by different letters in the same line are statistically different ($P<0.05$).

Table 2. Body and kidney weight of pups from dams exposed to $ZnCl_2$ and $HgCl_2$ during lactation

Parameters	Sal–Sal	Sal–Hg	Zn–Sal	Zn–Hg
Body weight gain (g)				
During Zn exposure (LD 3 to 7)	7.76 ± 0.17	8.16 ± 0.26	7.55 ± 0.38	8.01 ± 0.37
During Hg exposure (LD 8 to 12)	8.91 ± 0.16^{a}	3.11 ± 0.68^{b}	8.77 ± 0.35^{a}	3.07 ± 0.66^{b}
Body weight at LD 13 (g)	24.84 ± 0.34^{a}	19.45 ± 0.96^{b}	24.40 ± 0.71^{a}	19.19 ± 0.88^{b}
Kidney absolute weight (g)	0.26 ± 0.01^{a}	0.22 ± 0.01^{b}	0.26 ± 0.01^{a}	0.20 ± 0.01^{b}
Kidney relative weight (%)	1.01 ± 0.02	1.13 ± 0.04	1.02 ± 0.02	1.06 ± 0.05

Data are expressed as mean ± S.E.M. (n = 6–7) and the values followed by different letters in the same line are statistically different ($P<0.05$).

Table 3. Biochemical and metal determinations in kidney of pups from dams exposed to $ZnCl_2$ and $HgCl_2$ during lactation

Parameters	Sal–Sal	Sal–Hg	Zn–Sal	Zn–Hg
Biochemical determinations				
Urea (mg/dL)	36.89±1.05	32.47±1.41	37.27±4.18	30.86±2.82
Creatinine (mg/dL)	0.51±0.08	0.50±0.08	0.49±0.08	0.63±0.12
d-ALA-D activity*	6.74±0.53	7.04±0.49	6.74±0.41	7.23±0.38
Metal determinations				
Mercury levels (μg Hg/g)	0.22±0.02[a]	0.78±0.02[b]	0.26±0.05[a]	0.88±0.20[b]
Zinc levels (μg Zn/g)	18.93±1.90	18.22±2.10	23.34±4.39	22.17±5.16

Data are expressed as mean ± S.E.M. (n= 6–7 for biochemical determinations and 3–5 for metal determinations). The values followed by different letters in the same line are statistically different ($P<0.05$).

* Expressed as nmol PBG/ mg protein/ h.

Figures

Figure 1.

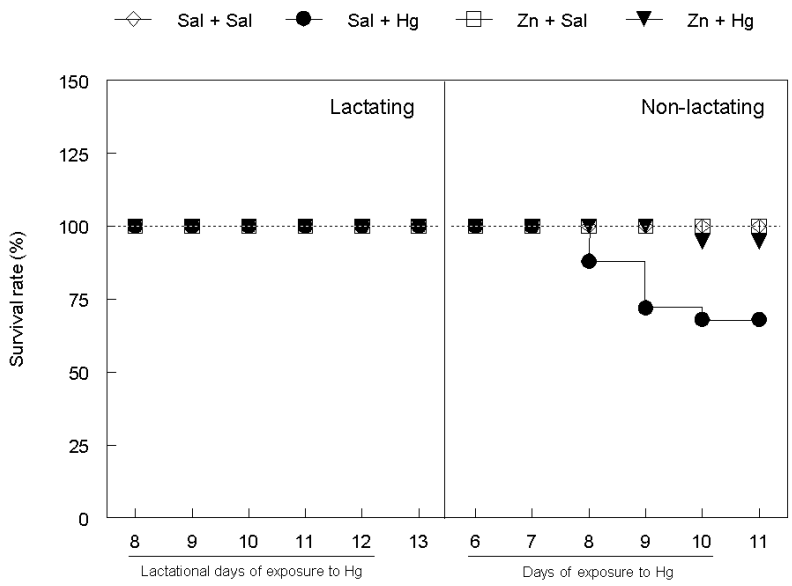


Figure 2a.

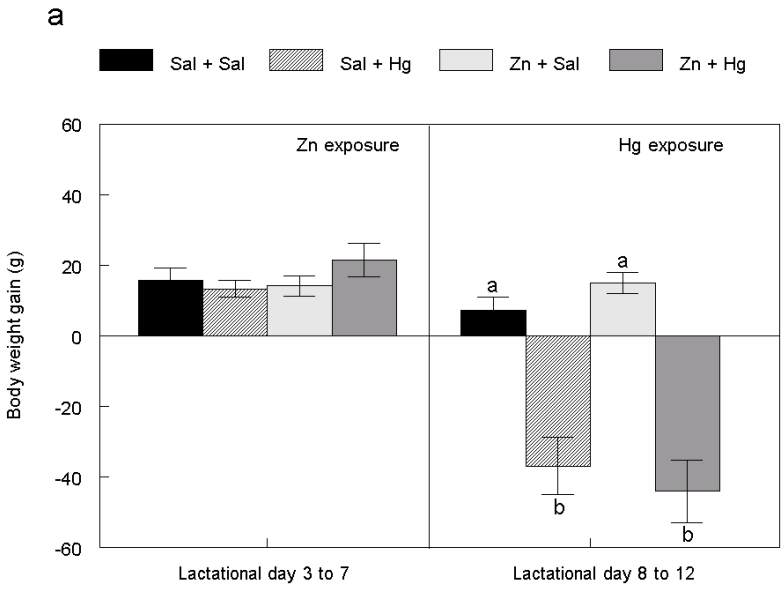
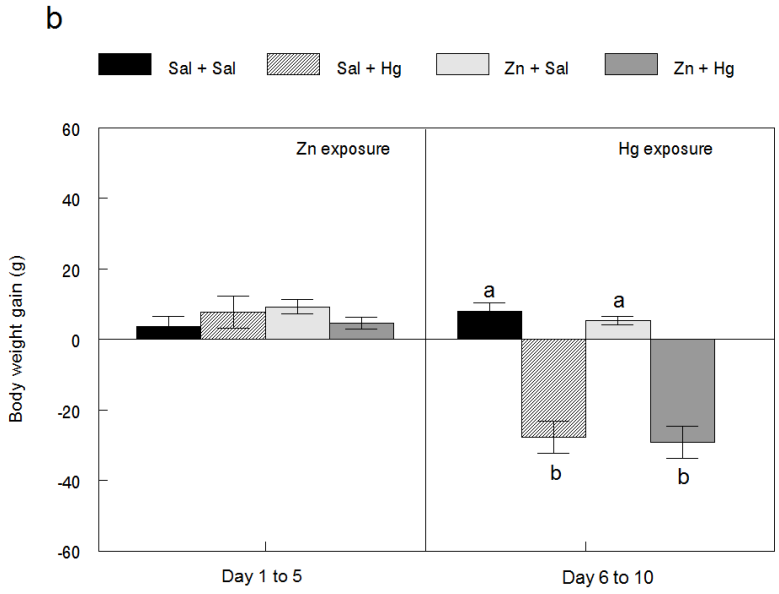


Figure 2b.



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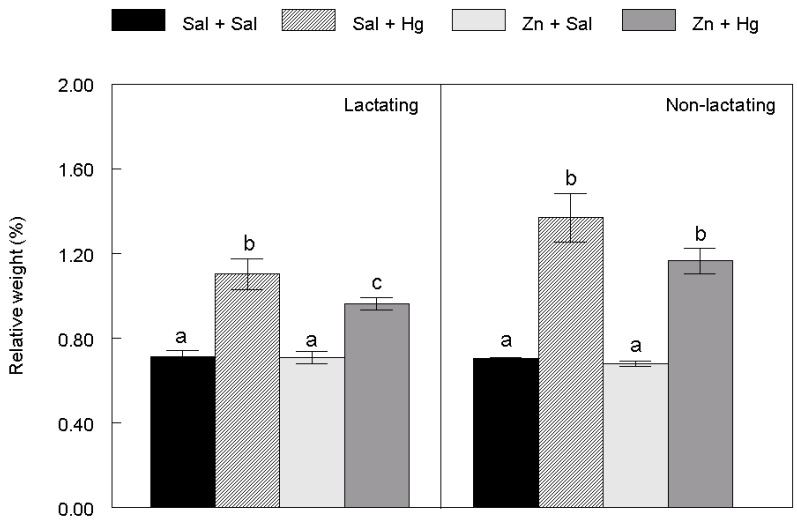


Figure 4a.

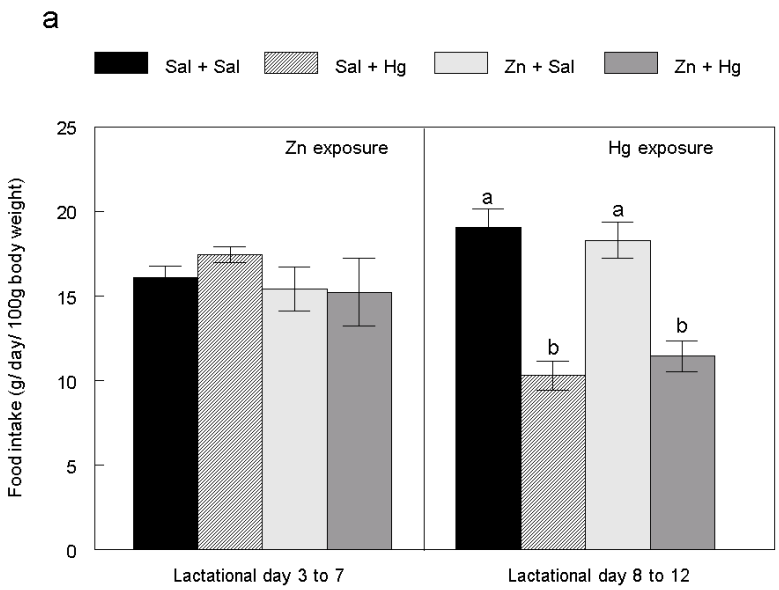
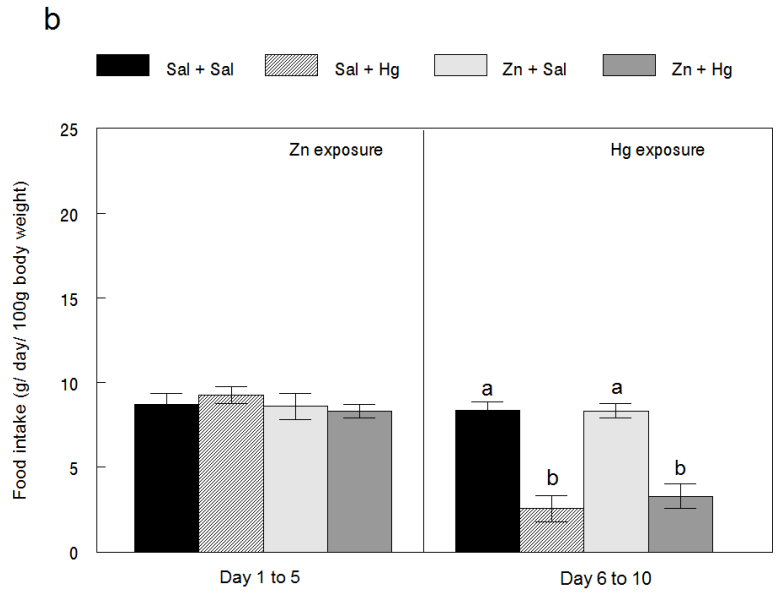


Figure 4b.



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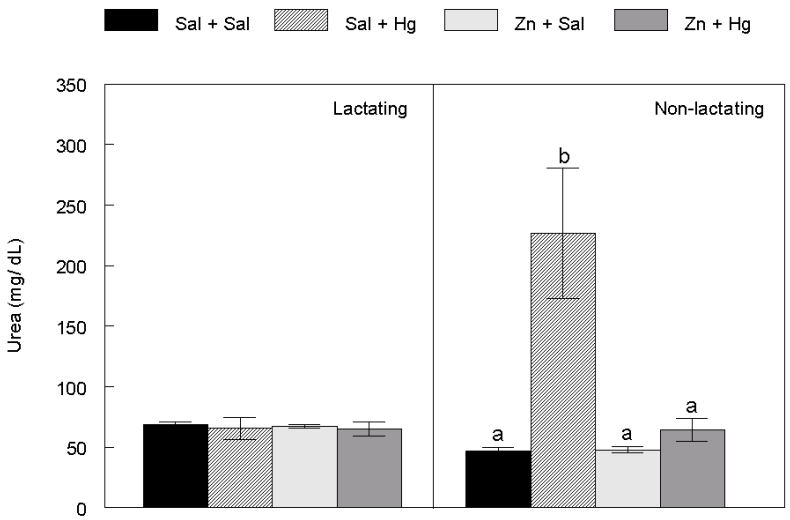

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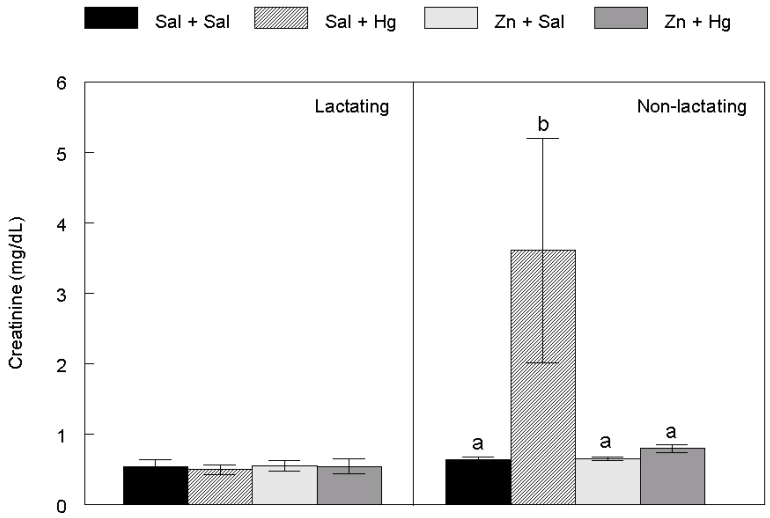


Figure 7.

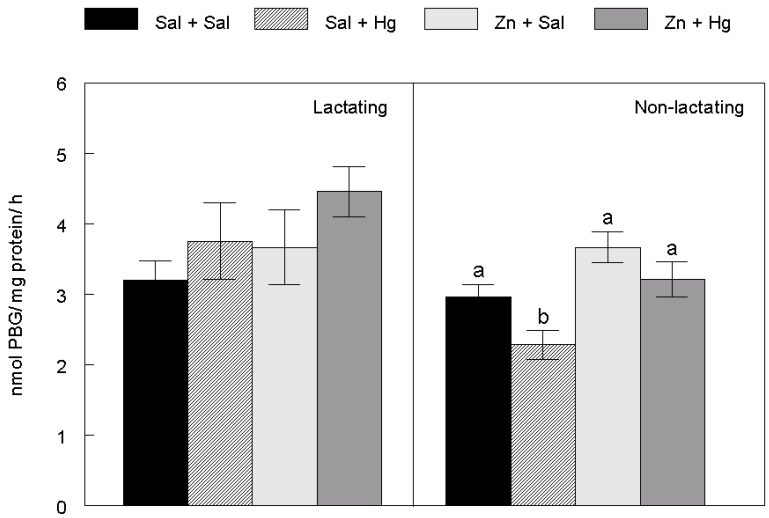


Figure 8a.

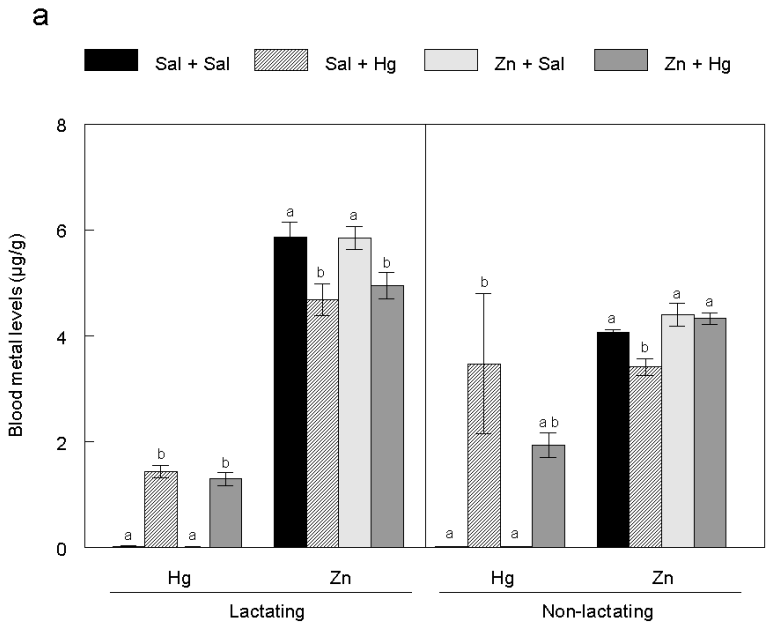


Figure 8b.

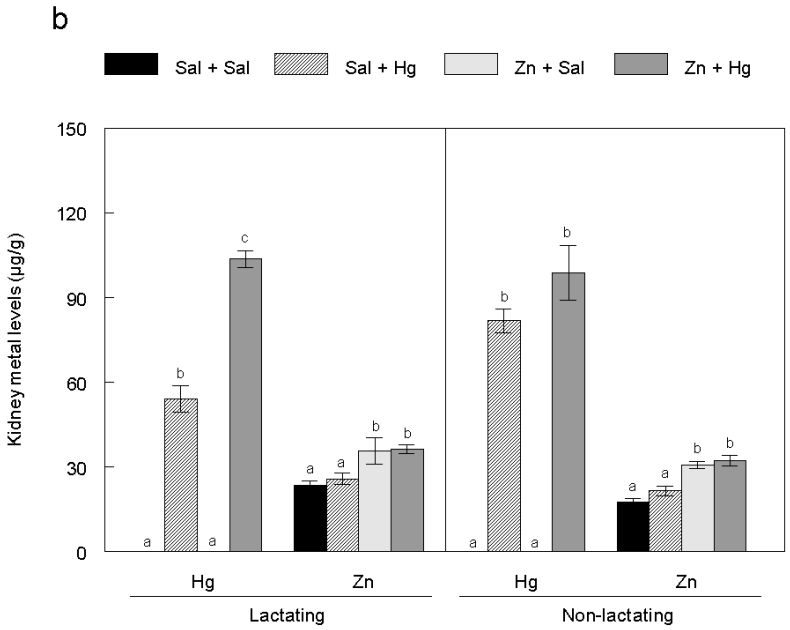

b
Sal + Sal
Sal + Hg
Zn + Sal
Zn + Hg
Kidney metal levels (µg/g)
150
120
90
60
30
0
a
b
a
c
a
a
b
b
a
b
a
b
a
a
b
b
Hg
Zn
Hg
Zn
Lactating
Non-lactating

Figure 9.

Plate 1–A (Sal–Sal)

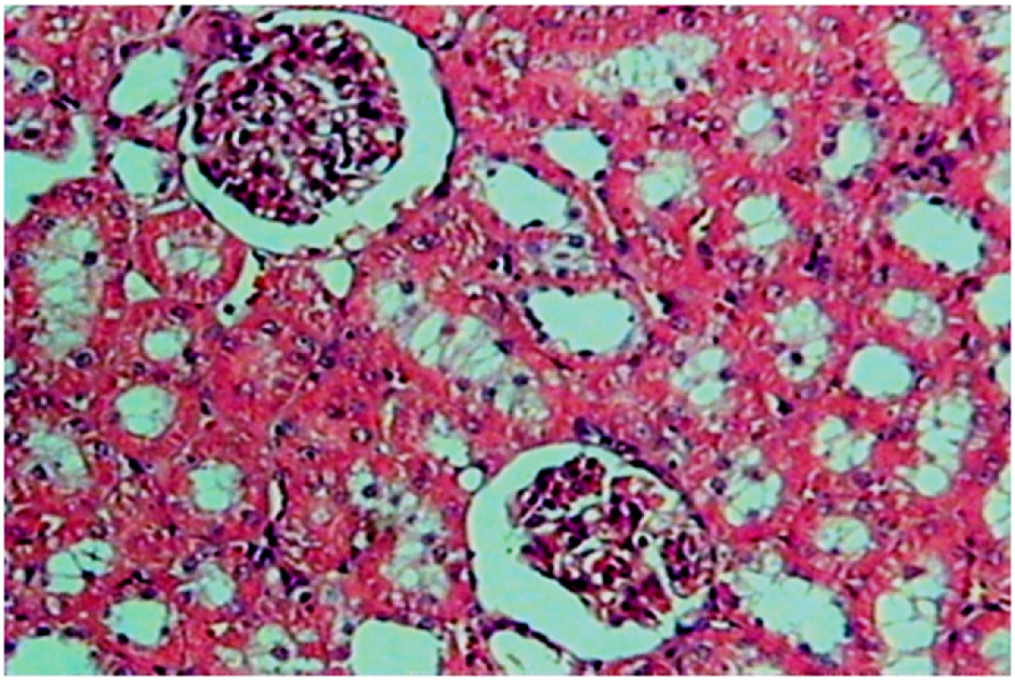


Plate 1–B (Sal–Hg)

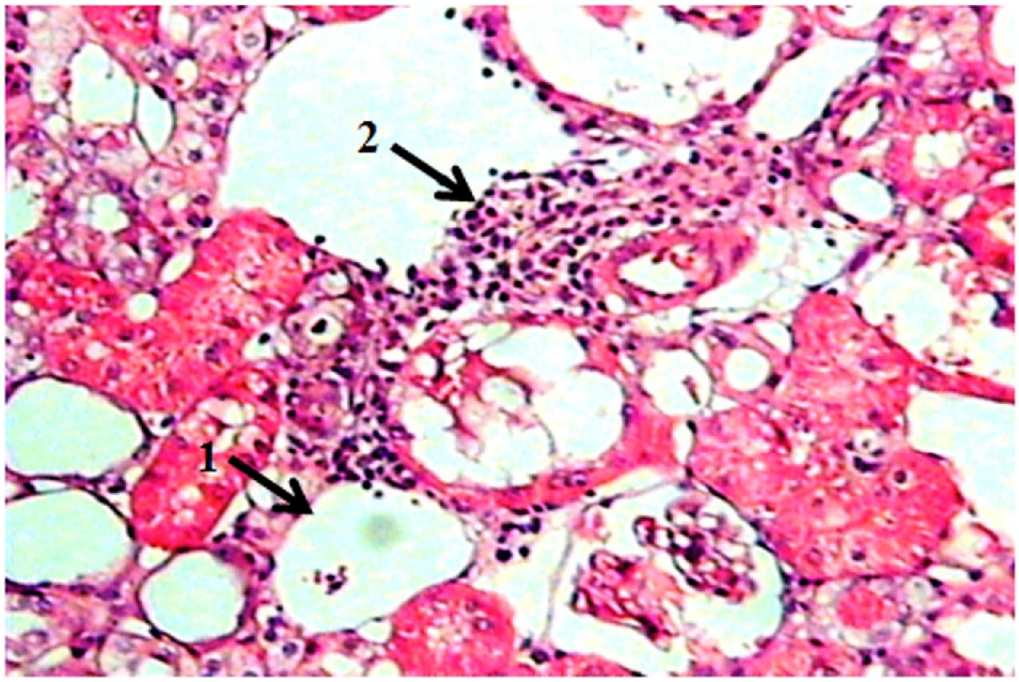


Plate 1–C (Zn–Sal)

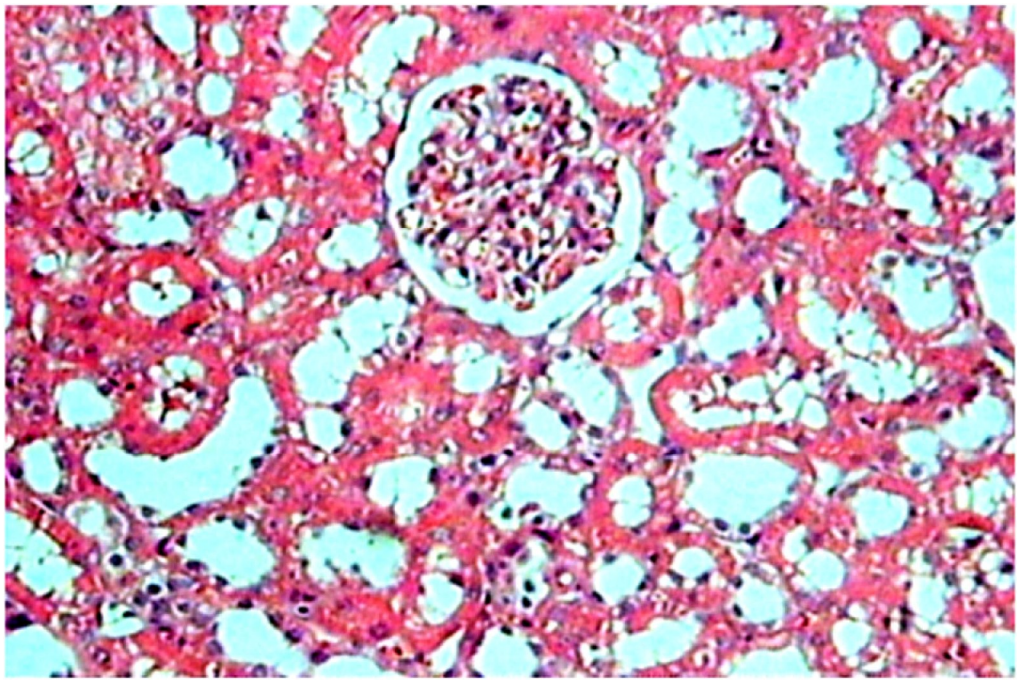


Plate 1–D (Zn–Hg)

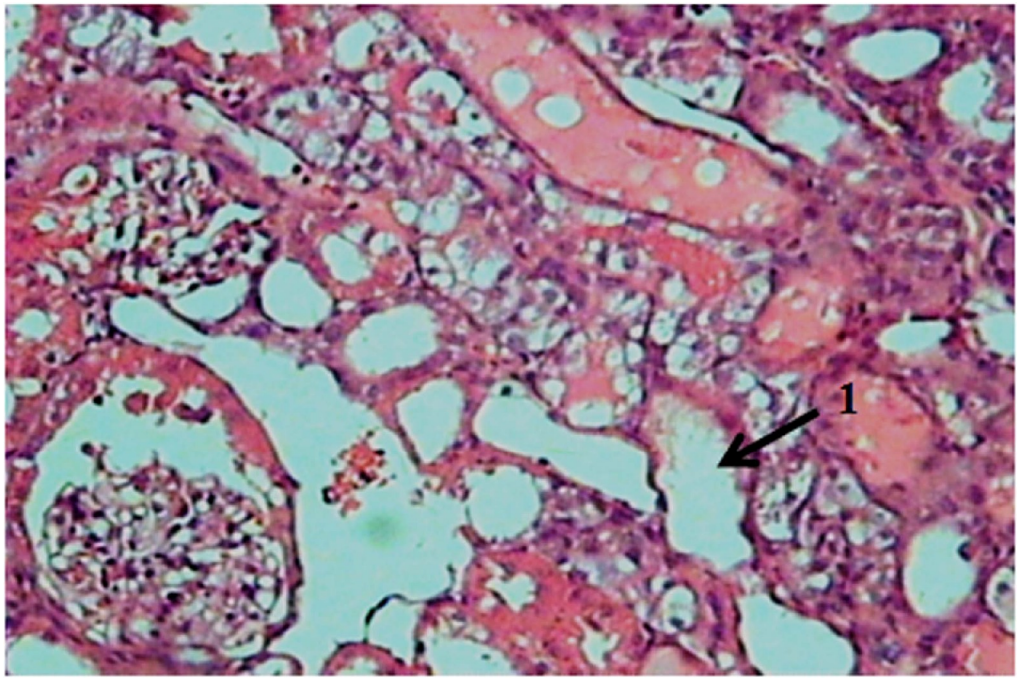


Plate 2–A (Sal–Sal)

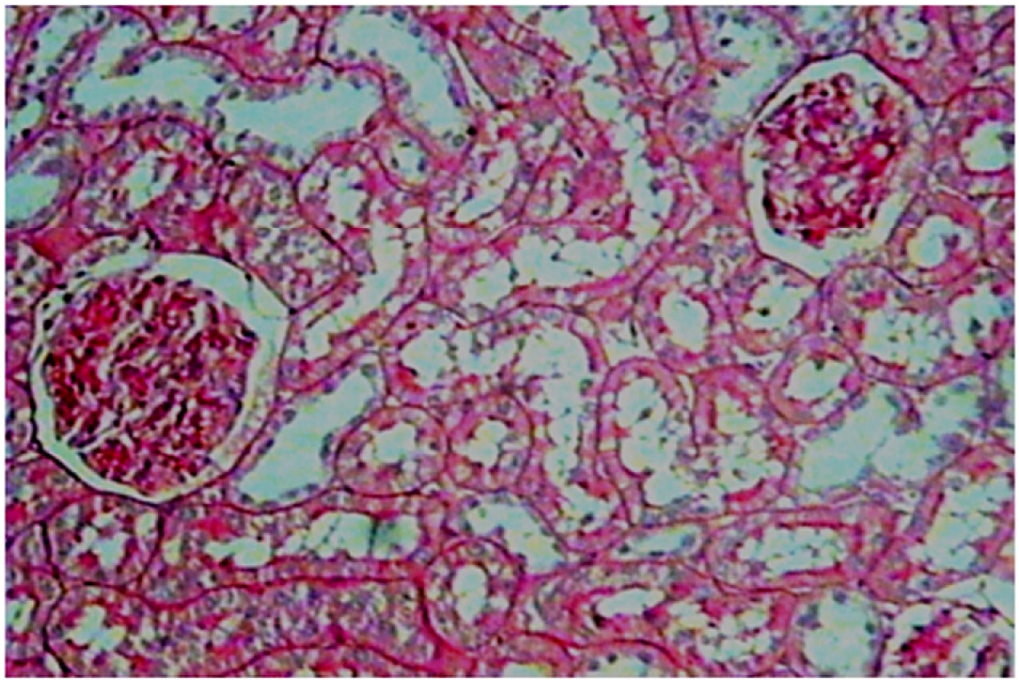


Plate 2–B (Sal–Hg)

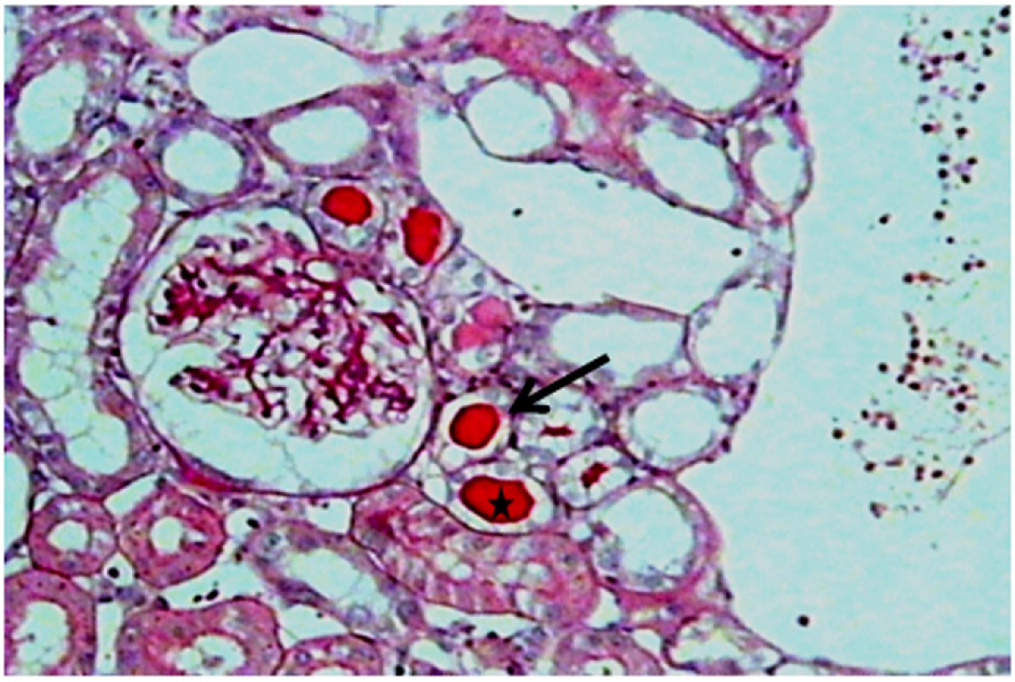


Plate 2–C (Zn–Sal)

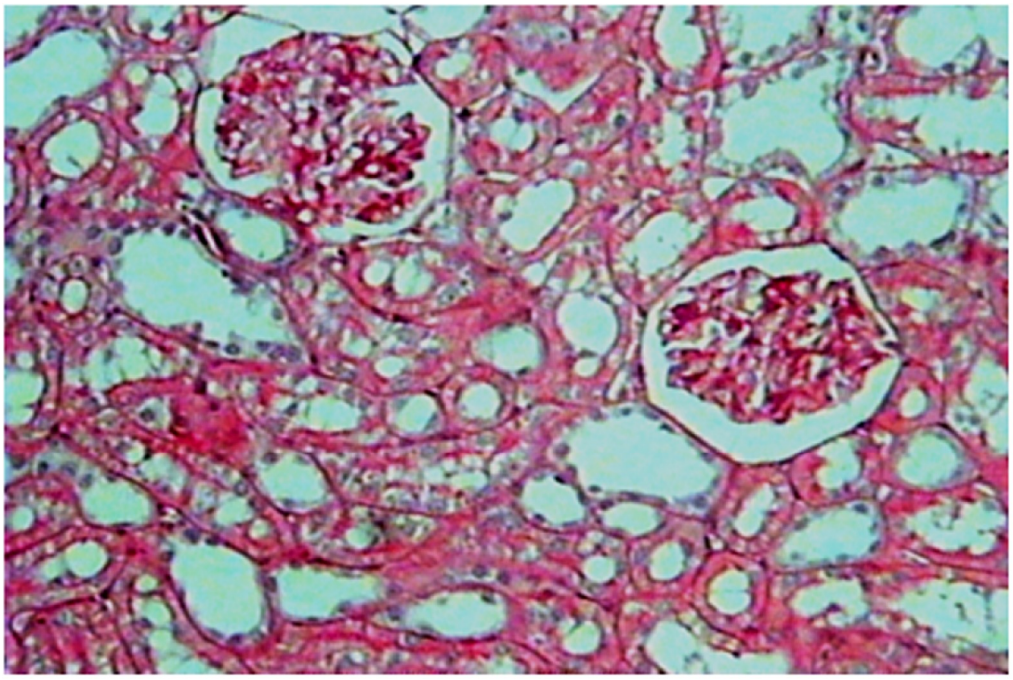


Plate 2–D (Zn–Hg)

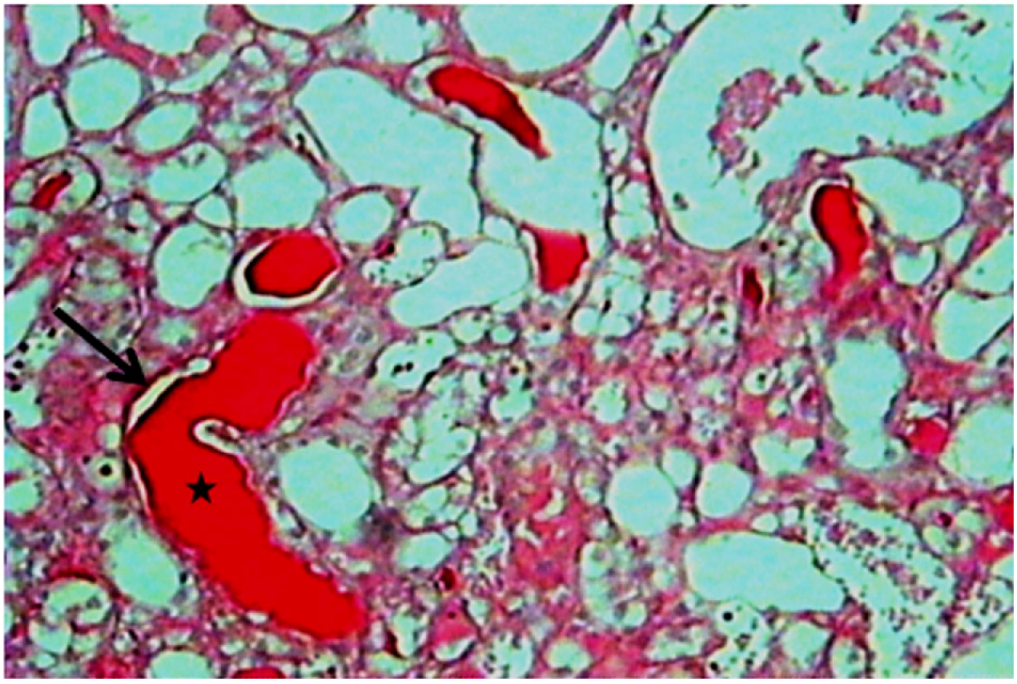


Plate 3–A (Sal–Sal)

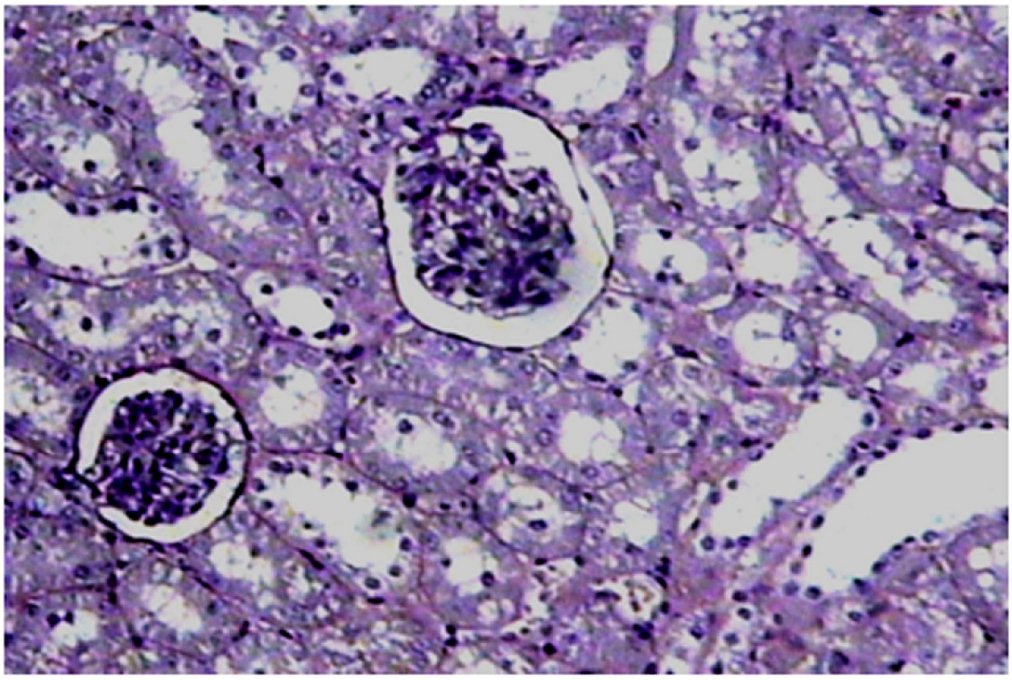


Plate 3–B (Sal–Hg)

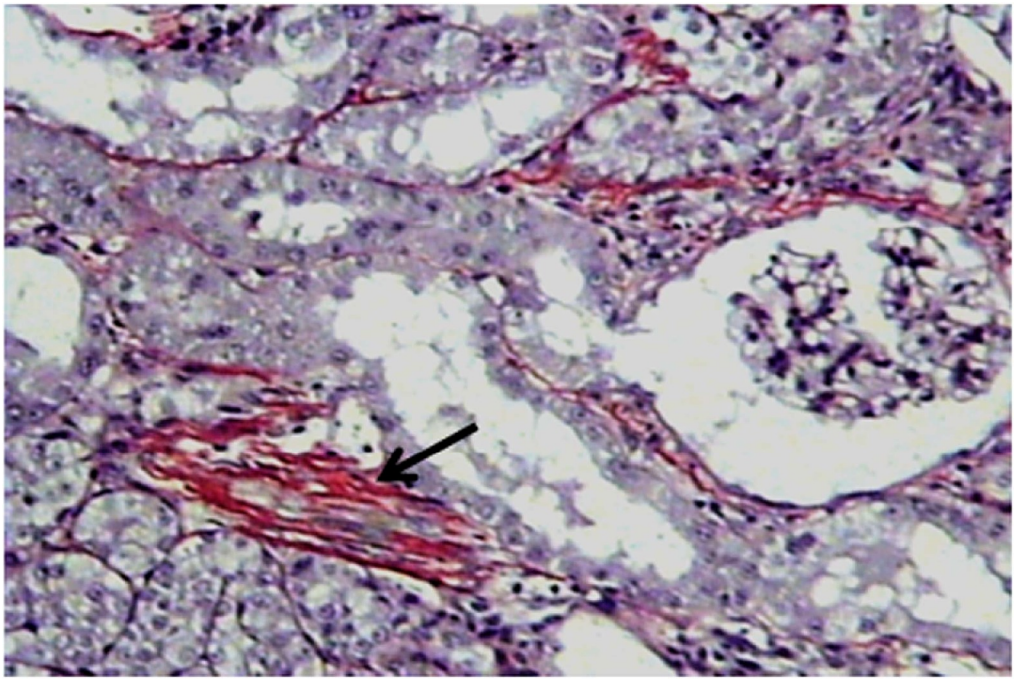


Plate 3–C (Zn–Sal)

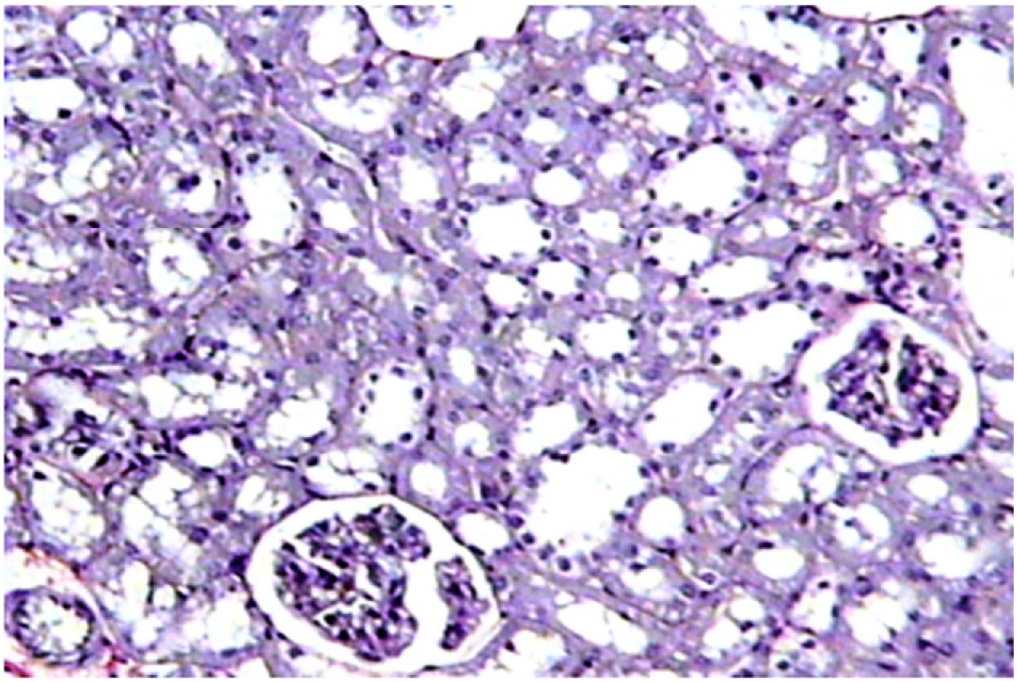
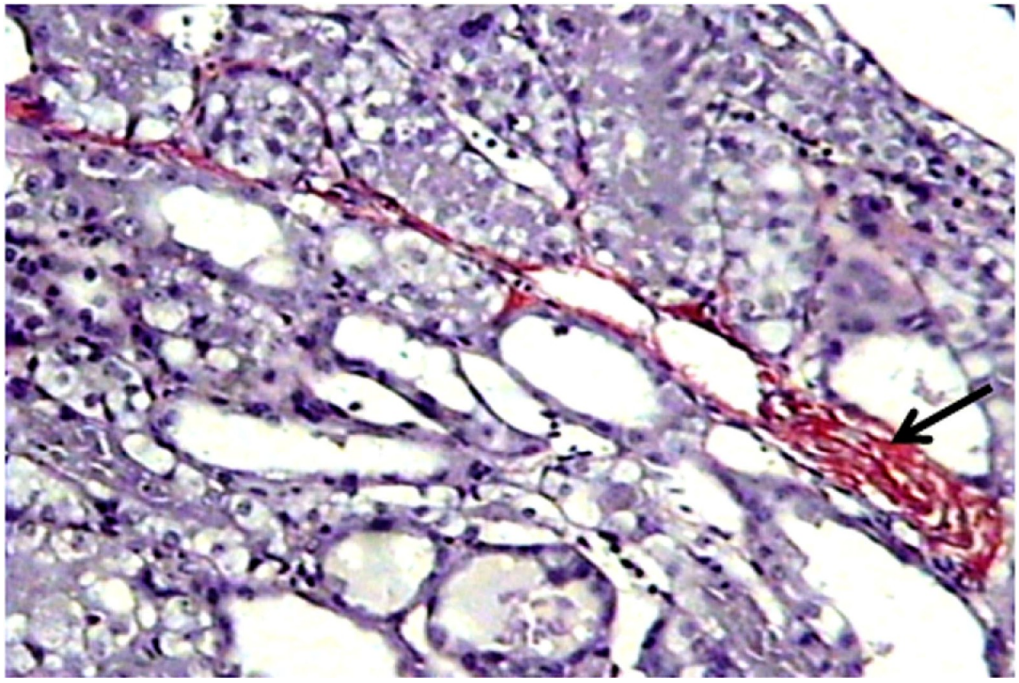


Plate 3-D (Zn–Hg)



3.2. Manuscrito II

Distinct response of lactating and non-lactating rats exposed to inorganic mercury on hepatic parameters

Alexandre M. Favero, Carina Franciscato, Cláudia S. Oliveira, Juliana S. F. Pereira, Sônia C. A. da Luz, Valderi L. Dressler, Erico M. M. Flores e Maria E. Pereira

Manuscrito submetido ao Periódico *Ecotoxicology and Environmental Safety*

Elsevier Editorial System(tm) for Ecotoxicology and Environmental Safety
Manuscript Draft

Manuscript Number: EES-11-153

Title: Distinct response of lactating and non-lactating rats exposed to inorganic mercury on hepatic parameters

Article Type: Regular Article

Keywords: δ-ALA-D; lactation; liver; mercuric chloride; pups; rats; zinc chloride.

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Abstract: The effects of $HgCl_2$ exposure on hepatic parameters in lactating and non-lactating rats and the potential preventive role of $ZnCl_2$ were evaluated. Animals received 27 mg/kg $ZnCl_2$ (or saline) for five consecutive days and 5 mg/kg $HgCl_2$ (or saline) for five subsequent days. Non-lactating rats exposed to $HgCl_2$ presented increased hepatic Hg levels. Lactating and non-lactating rats from Zn-Hg group showed increased hepatic Hg and Zn levels. Hepatic δ-ALA-D activity was increased in lactating rats from both $HgCl_2$-exposed groups. Non-lactating rats exposed to $HgCl_2$ presented increased AST activity and zinc pre-exposure partially prevented it. Lactating and non-lactating rats exposed to $HgCl_2$ presented decreased ALT activity. Lactating rats from Zn-Hg group presented hepatic histological alterations. Hg-exposed pups showed decreased liver weight and increased hepatic Hg levels. This study demonstrated that lactating rats presented distinct biochemical responses comparing to non-lactating rats exposed to $HgCl_2$ when hepatic parameters were evaluated.

Distinct response of lactating and non-lactating rats exposed to inorganic mercury on hepatic parameters

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Abstract

The effects of $HgCl_2$ exposure on hepatic parameters in lactating and non-lactating rats and the potential preventive role of $ZnCl_2$ were evaluated. Besides, it was assessed the effects of metals on pups exposed to them through maternal milk. Lactating and non-lactating rats received 27 mg/kg $ZnCl_2$ (or saline) for five consecutive days and 5 mg/kg $HgCl_2$ (or saline) for five subsequent days. Pups from Sal–Hg and Zn–Hg groups showed a decrease in absolute liver weight and an increase in liver Hg levels. Non-lactating rats exposed to $HgCl_2$ presented higher hepatic Hg levels than control rats. Lactating and non-lactating rats from Zn–Hg group presented an increment in hepatic Hg and Zn levels. Lactating rats from Sal–Hg and Zn–Hg groups presented an increase on hepatic d-aminolevulinic acid dehydratase (d-ALA-D) activity; in non-lactating rats, no differences were observed on this parameter. Non-lactating rats exposed to $HgCl_2$ presented a slight increase in plasma AST activity and previous exposure to $ZnCl_2$ partially prevented it. Lactating and non-lactating rats exposed to $HgCl_2$ presented plasma ALT activity significantly decreased and previous exposure to $ZnCl_2$ did not prevent this alteration. Histopathological alterations were observed in liver of lactating rats from Zn–Hg group. Summarizing, this study demonstrated that lactating rats presented distinct biochemical responses compared to non-lactating rats exposed to $HgCl_2$ when hepatic parameters were evaluated.

Keywords: d-ALA-D, lactation, liver, mercuric chloride, pups, rats, zinc chloride

1. Introduction

Mercury (Hg) is a naturally-occurring heavy metal that may enter the environment through natural phenomena (e.g. volcanic activity and erosion of mineral deposits) as well as from a wide range of anthropogenic activities such as mining, metal smelting, coal production, coal-fired power stations, residential heating systems, waste disposal/incinerators and chemical synthesis/use (Holmes et al., 2009).

Mercury is present in the environment in a number of forms including elemental mercury (Hg^0), inorganic salts (Hg^+ and Hg^{2+}) and as organic compounds (e.g. methyl-, ethyl and phenyl-mercury) (Clarkson, 1997; Goldman and Shannon, 2001; Counter and Buchanan, 2004). There are evidences that mercury can cause a wide range of toxic effects in humans, depending on the specific form of mercury, the exposure level and duration, and the age at which an individual is exposed (Holmes et al., 2009). Exposure during susceptible developmental periods, such as pregnancy and breastfeeding, may make women as well as developing fetuses and breast-fed infants vulnerable to the toxicity of mercury in its various forms.

Mercuric chloride ($HgCl_2$), an inorganic form of mercury, is mainly recognized for adversely affect kidneys (Weinberg et al., 1982a; 1982b; Goyer, 1995; Emanuelli et al., 1996; Clarkson, 1997; Peixoto and Pereira, 2007; Peixoto et al., 2007a; Franciscato et al., 2011), the central and peripheral nervous system (Chang and Hartmann 1972; Lakshmana et al., 1993; Aschner et al., 1994; El-Demerdash, 2001; Franciscato et al., 2009) and the reproductive ability (Vonburg, 1995). Hepatotoxicity induced by this metal has been suggested by several authors. It has been reported that $HgCl_2$-exposure induces histopathological alterations in the liver (Kumar et al., 2005; Sharma et al., 2000; 2002; 2007) and increases in aspartate and alanine aminotransferase (AST and ALT) activities (El-Demerdash, 2001; Kumar et al., 2005; Sener et al., 2007; Sharma et al., 2007). In addition, it has been demonstrated that hepatotoxic effects of $HgCl_2$ appear to be associated with mitochondrial damage, by inhibiting oxidative phosphorylation, leading to a failure in energy production and consequently to lethal hepatocyte injury (Palmeira and Madeira 1997).

d-Aminolevulinate dehydratase (δ-ALA-D), an enzyme that participates in the second step of heme biosynthesis (Jaffe, 1995), is an important biomarker of toxicity for many xenobiotics because of potential sites of inhibition, including chemical modification of the cysteinyl residues (Jaffe et al., 1995; Barbosa et al., 1998; Farina et

al., 2001, 2002) and release of the prosthetic Zn^{+2} (Emanuelli et al., 1998; Vieira et al., 2000). Particularly, hepatic d-ALA-D has been assumed to be a target for $HgCl_2$-exposure in both young (Rocha et al., 1995; Peixoto et al., 2003; 2007a; Franciscato et al., 2011) and adult experimental animals (Emanuelli et al., 1996).

During lactation several physiological conditions are changed, such as increase in blood and plasma volume, decrease in total plasma protein, and increases in cardiac output and blood flow through organs as a response to the increasing demand from the young during this period (Hanwell and Linzell, 1973; Suzuki et al., 1993). These changes may certainly influence the distribution and elimination of chemical compounds (Hallén et al., 1996; Houpert et al., 1997, Sundberg et al., 1998). Indeed, the Hg biological half-time is around 40% lower both in humans and animals exposed to methyl mercury (Greenwood et al., 1978) and around 50% lower in animals exposed to inorganic mercury (Prester et al., 1994; 1997) during lactation. Magos et al. (1980) demonstrated that lactation delayed the onset of weight loss, shortened the time between the end of treatment and the onset of weight gain, accelerated the elimination of mercury from the whole body and prevented the development of severe co-ordination disorders after methyl mercury intoxication. Recently, it was demonstrated that $HgCl_2$-induced nephrotoxicity differs considerably between lactating and non-lactating rats (Favero et al., submitted). Even though organ distribution and toxicity of inorganic mercury have been studied by numerous investigators, differences in susceptibility to the $HgCl_2$-induced hepatotoxicity related to the lactation period are limited in the literature.

Based on the above, the objective of this study was to compare the effects of $HgCl_2$ exposure on hepatic d-ALA-D activity and on biochemical parameters indicative of hepatotoxicity in lactating and non-lactating rats as well as the potential preventive role of $ZnCl_2$. Additionally, the present work provides data on the effects of metals on pups exposed to them exclusively via maternal milk.

2. Materials and methods

2.1. Chemicals

Mercuric chloride ($HgCl_2$), zinc chloride ($ZnCl_2$), sodium chloride (NaCl), potassium phosphate monobasic (KH_2PO_4) and dibasic (K_2HPO_4), absolute ethanol, ether, sodium hydroxide (NaOH), trichloroacetic acid (TCA), nitric acid (HNO_3),

sulfuric acid (H_2SO_4), o-phosphoric acid, perchloric acid and glacial acetic acid were purchased from Merck (Darmstadt, Germany). d-Aminolevulinic acid (d-ALA), bovine serum albumin and Coomassie brilliant blue G were obtained from Sigma (St. Louis, MO, USA); ρ-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han., Germany). The kits for determination of glucose levels and LDH (lactic dehydrogenase), ALT (alanine aminotranferase) and AST (aspartate aminotranferase) activities were acquired from LABTEST (Lagoa Santa/ MG/ Brazil).

2.2. Animals

Adult female *Wistar* rats (pregnant and non-pregnant), 90 days old, obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12-h light/dark cycle and at a controlled temperature (22 ± 2ºC). Pregnant rats were allowed to deliver and wean their pups until lactational day (LD) 13. One day after the birth, litters were culled randomly to eight pups each. The animals had free access to water and commercial food (GUABI, RS, Brazil). The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Exposure to metals

Lactating and non-lactating rats were distributed on a random basis into four groups of six to seven animals each and housed in individual standard polypropylene plastic cages (41 x 34 x 18 cm). Animals were subcutaneously (s.c.) exposed to 0.9% NaCl (saline solution) or metals dissolved in saline solution at a volume of 1 mL/kg body weight (b.w.) following the experimental design:

I - Exposure protocol of lactating rats

Group 1 (Sal–Sal): lactating rats received saline for ten consecutive days (LD 3 to 12) to serve as control.

Group 2 (Sal–Hg): lactating rats received saline for five consecutive days (LD 3 to 7). On five subsequent days (LD 8 to 12) animals were daily exposed to 5 mg/kg $HgCl_2$.

Group 3 (Zn–Sal): lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals received saline.

Group 4 (Zn–Hg): lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals were daily exposed to 5 mg/kg $HgCl_2$.

The suckling pups were exposed to metals exclusively through maternal milk. It is important to mention that the transport of inorganic mercury into milk was previously demonstrated in the literature (Sundberg et al., 1991; Yoshida et al., 1994).

II - Exposure protocol of non-lactating rats

Group 1 (Sal–Sal): non-lactating rats received saline for ten consecutive days (days 1 to 10) to serve as control.

Group 2 (Sal–Hg): non-lactating rats received saline for five consecutive days (days 1 to 5). On five subsequent days (days 6 to 10) the animals were daily exposed to 5 mg/kg $HgCl_2$.

Group 3 (Zn–Sal): non-lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals received saline.

Group 4 (Zn–Hg): non-lactating rats received 27 mg/kg $ZnCl_2$ for five consecutive days. On five subsequent days the animals were daily exposed to 5 mg/kg $HgCl_2$.

Animals were weighed immediately before exposure to metals to adjust the dose. The doses, exposure route and time period that lactating (LD 3 to 12) and non-lactating rats were exposed to metals were chosen according to previous studies performed by our research group (Peixoto et al. 2003; 2007a; 2007b; 2008; Peixoto and Pereira, 2007; Franciscato et al., 2009; 2011). These studies demonstrated that 5 mg/kg/day $HgCl_2$ caused toxic effects which were prevented by 27 mg/kg/day $ZnCl_2$ in suckling rats directly exposed to metals and killed 24h after the end of exposure period. All animals were observed daily throughout the study for mortality and signs of toxicity. Water and food consumption of lactating and non-lactating rats were monitored daily during the period of exposure to metals.

2.4. Tissue preparation

Twenty-four hours after the last administration of $HgCl_2$, lactating (and their pups) and non-lactating rats were weighed, anesthetized with ether and euthanized by decapitation. Blood samples were collected in tubes with heparin and centrifuged at 2000×*g* for 10 min at 4°C to obtain the plasma. The liver was removed and weighed. For d-ALA-D activity assay, the liver was quickly placed on ice and homogenized in 7

volumes of NaCl (150 mM, pH 7.4) with 10 up-and-down strokes at ~1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged at 8000×*g* for 30 min at 4°C and the supernatant fraction was used in the enzyme assay. For determination of metal Hg and Zn levels, tissue samples were placed into vials and then frozen at -20 ºC until analysis. For histological examination, liver was fixed in 10% formalin solution for 48 h, routinely processed, embedded in paraffin and sectioned. The sections were cut 4 µm-thick for histopathological examination.

2.5. Biochemical determinations

2.5.1. Hepatic d-ALA-D activity

The enzymatic activity was assayed according to the method of Sassa (1982) by measuring the rate of product (porphobilinogen - PBG) formation, except that 76 mM sodium phosphate buffer (pH 6.8) and 2.2 mM d-ALA were used. The incubation was initiated by adding 200 µL of 8000×*g* supernatant of the liver homogenate and was carried out for 40 min at 39 ºC. The reaction was stopped by the addition of TCA 10% containing $HgCl_2$ 0.05 mol/L and the PBG was measured with Ehrlich's reagent at 555 nm, using the molar absorption coefficient of 6.1 x 10^4 for Ehrlich-PBG salt. The specific enzymatic activity was expressed as nmol of PBG formed per hour per mg protein.

2.5.2. Plasma ALT and AST activities

The ALT activity was determined by the Reitman and Frankel (1957) method in a medium containing buffer 55.8 mmol/L pH 7.4, a-ketoglutaric acid 1.67 mmol/L, L-alanine 83.3 mmol/L, and sodium azide 12.8 mmol/L and 25 µL of sample incubated at 37°C for 30 min. The reaction was stopped by adding HCl 0.45 mol/L. The color reactive (2,4-dinitrophenylhydrazine 0.45 mmol/L) was added and the medium was incubated for 20 min at room temperature. The color was intensified by NaOH 0.33 mol/L and the absorbance was determined at 505 nm. The activity (in U/mL) was calculated by comparison with a calibration curve utilizing sodium pyruvate as standard. The AST activity was determined similarly to ALT enzyme, except that L-aspartic acid 83.3 mmol/L was used as substrate and that the medium was incubated at 37ºC for 60 min.

2.5.3. Plasma LDH activity

The LDH activity (inU/L) was determined by the formation of NADH. The medium containing buffer 200 mmol/L pH 8.2, lactic acid 260 mmol/L, sodium azide 7.7 mmol/L and 25 mL of plasma was incubated at 37ºC for 2 min. After this period, the color reactive (INT 0.64 mmol/L, NAD^+ 1.2 mmol/L, phenazine 0.26 mmol/L and sodium azide 1.23 mmol/L) was added and the medium was incubated for another 5 min at 37ºC. The reaction was stopped by adding HCl 200 mmol/L and the tubes remained at room temperature for 5 min until quantification of absorbance at 500nm.

2.5.4. Glucose

The quantification of plasma glucose was carried out by measuring the product formed in a reaction medium containing phosphate buffer 9.9 mmol/L pH 7.4, phenol 9.9 mmol/L, glucose oxidase (= 12,000 U/L), peroxidase (= 1,000 U/L) and 4-amino-antipyrine 0.4 mmol/L. The incubation was started by adding 10 µL of sample and realized at 37°C for 10 min. The product formed from the complete oxidation of the glucose is a red-violet quinoneimine, whose absorbance was measured at 500 nm.

2.5.5. Protein determination

Protein concentrations were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.6. Metals determinations

Samples weighing around 250 mg each were transferred to quartz vessels. Concentrated HNO_3 (6 mL) was added to each vessel, which was capped and placed into the microwave oven. Samples were digested using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280°C, Anton Paar, Graz, Austria). The microwave oven operated at 1400 W for 30 min and at 0 W for 20 min. The last step was used for cooling. After digestion samples were diluted with water to 25 mL and transferred to graduated polypropylene vials. Colorless and clear solutions were obtained after the digestion step. Spike recovery tests and biological certified reference material (SRM NIST 1577, bovine liver) were carried out to validate the results. Blanks were run and analyzed after each ten measurements in order to check eventual memory effects for all elements.

Metal analyses were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes. A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for Hg determinations. A deuterium background corrector was used for all the determinations. Hollow cathode lamps used were operated at 4 mA for both metals. Wavelength was set at 324.8, 213.9 and 253.7 nm and the spectral band pass at 1.2, 0.5 and 0.5 nm, respectively. Integrated absorbance (peak area) was used for all measurements. Heating program for Zn was carried out according to the recommendations of manufacturer. For Hg determination, 3 M HCl and 0.25% m/v $NaBH_4$ solutions were used as acid medium and reductant, respectively. Argon was used as purge gas. Results for all elements determined were periodically evaluated by measurements of analytical standards (each 10 measurements) and also by the digests analysis of certified reference material SRM NIST 1577 (each 3 h of measurements). If the result for standards checking presented a bias higher than 5% a recalibration procedure was performed.

2.7. Histopathological examination

Conventional histopathology was performed on tissue fixed in 10% formalin solution and embedded in paraffin: 4 µm-thick sections were stained with hematoxylin–eosin (H&E) for microscopic observations. The histological preparations were examined with a light microscope (Bioval L2000C).

2.8. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's t-test when appropriate. Comparisons among all group treatment means were made. Different letters were used to indicate significant differences among groups. Lactating and non-lactating rats were independently analyzed. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Effects of exposure to metals in lactating and non-lactating rats

3.1.1. Liver weight

One-way ANOVA revealed that absolute liver weight of lactating rats was significantly affected by exposure to metals [$F(3,23)= 5.65$, $P<0.01$]. Animals from both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented lower absolute liver weight (around 20%) when compared to Zn–Sal group ($P<0.05$, Duncan's multiple range test). In contrast, this parameter was not altered in any group of non-lactating rats. The relative liver weight was altered neither for lactating nor for non-lactating rats (Table 1).

3.1.2. Biochemical determinations

3.1.2.1. Hepatic d-ALA-D activity

Hepatic d-ALA-D activity was significantly affected by exposure to metals in lactating rats [$F(3,23)= 6.70$, $P<0.001$]. Post-hoc comparisons showed that lactating rats from both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented an increase (around 50%) in enzyme activity when compared to the other groups (Sal–Sal and Zn–Sal) ($P<0.05$, Duncan's multiple range test) (Fig. 1). Hepatic d-ALA-D activity was not changed by exposure to metals in non-lactating rats.

3.1.2.2. Plasma ALT and AST activity

One-way ANOVA revealed that ALT activity was significantly changed by exposure to metals in both lactating [$F(3,23)= 8.83$, $P<0.001$] and non-lactating [$F(3,22)= 19.40$, $P<0.001$] rats. Post-hoc comparisons showed that lactating and non-lactating rats exposed to $HgCl_2$ presented ALT activity significantly lower than their respective control groups (around 48 and 52%, respectively). Previous exposure to $ZnCl_2$ did not prevent the alteration induced by $HgCl_2$ on ALT activity (Table 2).

One-way ANOVA revealed that AST activity was significantly modified by metals only in non-lactating rats [$F(3,22)= 3.32$, $P<0.05$]. Post-hoc comparisons showed that non-lactating rats exposed to $HgCl_2$ presented a slight increase in AST activity (around 20%) when compared to the control group. Previous exposure to $ZnCl_2$ partially prevented this increase ($P<0.05$, Duncan's multiple range test). AST activity was not changed by exposure to metals in lactating rats (Table 2).

3.1.2.3. Plasma LDH activity

LDH activity remained unchanged after exposure to metals in both lactating and non-lactating rats (Table 2).

3.1.2.4. Glucose

Glucose levels were not modified by exposure to metals in both lactating and non-lactating rats (Table 2).

3.1.3. Hepatic Hg and Zn levels

One-way ANOVA revealed significant effects of exposure to metals on hepatic Hg [$F(3,12)= 11.27$, $P<0.001$] and Zn [$F(3,8)= 5.42$, $P<0.05$] levels in lactating rats. The lactating rats exposed to both metals (Zn–Hg group) presented a significant increase in hepatic Hg and Zn levels when compared to the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 2a and b). Also, comparison between Sal–Sal and Sal–Hg groups revealed that hepatic Hg levels of lactating rats exposed exclusively to $HgCl_2$ was significantly higher than the controls one (Student's *t* test: $P<0.05$).

One-way ANOVA revealed significant effects of exposure to metals on hepatic Hg [$F(3,10)= 5.12$, $P<0.05$] and Zn [$F(3,10)= 12.18$, $P<0.001$] levels in non-lactating rats. The non-lactating rats exposed to $HgCl_2$ presented a significant increase in hepatic Hg levels when compared to the control group ($P<0.05$, Duncan's multiple range test). $ZnCl_2$ pre-exposure was not able to prevent the alteration induced by $HgCl_2$ on hepatic Hg levels (Fig. 2a). The non-lactating rats exposed to both metals (Zn–Hg group) presented a significant increase in hepatic Zn levels when compared to the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 2b).

3.1.4. Histopathological examination

Light microscopical observations on the histological structures of liver from lactating rats exposed to both metals (Zn–Hg group) showed alterations including loss of lobular architecture with hepatocytes enlarged, swollen and finely vacuolated. Dilated mid-zonal sinusoids are seen in focal areas (Fig. 4b). On the other hand, the hepatic tissue of lactating rats from Sal–Sal, Sal–Hg and Zn–Sal groups and non-lactating rats from all groups showed normal histology. Sections of hepatic tissue of

these animals revealed normal hepatic parenchyma. Hepatocytes showed preserved structure (Fig. 4a and data not shown).

3.2. Effects of exposure to metals in suckling pups

3.2.1. Liver weight

One-way ANOVA revealed that absolute liver weight of suckling pups was significantly altered by exposure to metals [$F(3,23)= 13.08$, $P<0.001$]. Pups from Sal–Hg and Zn–Hg groups showed a decrease in the absolute liver weight of around 25% when compared to the control group ($P<0.05$, Duncan's multiple range test). Relative liver weights were similar among groups (Table 3).

3.2.2. Biochemical determinations

Hepatic d-ALA-D, plasma ALT, AST and LDH activities and glucose levels were not altered by exposure to metals in suckling pups (Table 3).

3.2.3. Metal determinations

One-way ANOVA revealed significant effects of exposure to metals on hepatic Hg [$F(3,8)= 10.39$, $P<0.01$] and Zn [$F(3,12)= 6.62$, $P<0.05$] levels in pups. Suckling pups from Sal–Hg and Zn–Hg groups presented a significant increase in hepatic Hg levels when compared to the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 3a). In addition, pups from Zn–Sal and Zn–Hg groups presented a significant increase in hepatic Zn levels when compared to the other groups ($P<0.05$, Duncan's multiple range test) (Fig. 3b).

3.2.4. Histopathological examination

There were no histopathological alterations in any group of suckling pups (data not shown).

4. Discussion

The objective of this study was to compare the effects of $HgCl_2$ exposure on hepatic d-ALA-D activity and biochemical parameters indicative of hepatotoxicity in lactating and non-lactating rats and the potential preventive role of $ZnCl_2$. In addition, it was assessed the effects of metals on pups exposed to them through maternal milk. Our results demonstrated that exposure to $HgCl_2$ increased hepatic Hg levels and decreased ALT activity in both lactating and non-lactating rats; activated d-ALA-D enzyme from liver of lactating rats; and increased AST activity, a marker of hepatotoxicity, only in non-lactating rats.

In this study both lactating and non-lactating rats presented an increase in hepatic Hg levels after exposure to $HgCl_2$. Interestingly, it was demonstrated for the first time that previous exposure to $ZnCl_2$ enhanced hepatic Hg accumulation in lactating rats. In accordance, histopathological examination showed alterations including loss of lobular architecture, hepatocellular degeneration (with hepatocytes enlarged, swollen and finely vacuolated) and sinusoidal dilatation exclusively in the liver of lactating rats from Zn–Hg group. Earlier reports have demonstrated that $HgCl_2$ exposure induces various histopathological alterations in the liver of experimental animals, including centrilobular necrosis, degranulation, cytoplasmic vacuolization, karyolysis, karyorhexis and pronounced sinusoidal spaces with granular hepatocytoplasm (Kumar et al., 2005; Sharma et al., 2000, 2002; 2007). Despite the histopathological changes observed, no differences on biochemical parameters related to Hg-exposure were observed in lactating rats exposed to Hg alone or in association with Zn. Lactating rats from both groups exposed to $HgCl_2$ (Sal–Hg and Zn–Hg) presented a similar increase on hepatic d-ALA-D activity (around 40%), which was not prevented by pre-exposure to $ZnCl_2$. The effect of $HgCl_2$ on hepatic d-ALA-D activity from lactating rats cannot be solely explained by Hg levels in this tissue, since Hg levels were significantly higher in the liver of lactating rats from Zn–Hg group. Moreover, d-ALA-D activity of non-lactating rats from both groups exposed to $HgCl_2$ was not affected despite the elevated hepatic Hg levels.

The d-ALA-D results in liver of lactating rats were unexpected. Several lines of evidence have demonstrated that hepatic d-ALA-D is sensitive to the inhibitory action of inorganic mercury poisoning (Rocha et al., 1995; Emanuelli et al., 1996; Peixoto et

al., 2003; 2007a; Franciscato et al., 2011), as also shown in the *in vitro* studies (Rocha et al., 1995; Peixoto et al., 2004). Interestingly, methyl mercury, an organic form of Hg, increased d-ALA-D (around 48%) activity in primary hepatocyte culture from *H. malabaricus*, a wild fish collected in Amazon basin (Filipak Neto et al., 2008). Regardless of the mechanism underlying the increased enzyme activity after $HgCl_2$ exposure, the present data clearly demonstrate that the behavior of hepatic d-ALA-D of lactating rats is markedly distinct from that observed in non-lactating animals.

Aminotransferases and LDH enzymes are sensitive indicators of hepatocellular damage (Meyer et al., 1992; Devlin, 1997). The LDH activity was not affected by Hg-exposure in both lactating and non-lactating rats in the present study. In contrast, a 20% increment in plasma AST activity of non-lactating rats were observed 24h after the end of Hg-exposure period and this effect was partially prevented when the animals were pre-exposed to $ZnCl_2$, indicating Zn pre-exposure as an important preventive treatment against mercury-induced hepatotoxicity. An increase in AST activity related to $HgCl_2$ exposure was previously demonstrated by several authors (El-Demerdash, 2001; Kumar et al., 2005; Sener et al., 2007; Sharma et al., 2007). On the other hand, it was observed a decrease in plasma ALT activity of lactating (around 48%) and non-lactating (around 52%) rats exposed to $HgCl_2$ and this effect was not prevented when the animals were pre-exposed to $ZnCl_2$. In accordance, Peixoto and Pereira (2007) showed a reduction of 40% in ALT activity of young rats exposed to $HgCl_2$. Recently, Franciscato et al. (2011) demonstrated that the effect of Hg on reducing ALT activity continues for a long time after cessation of metal intoxication. In that study, rats more sensitive to mercury exposure showed a decrement of 66.2% in ALT activity even 21 days after the end of exposure period. The effect of Hg on ALT activity cannot be defined as being a hepatotoxicity event since liver lesion is feature by the increase, and not decrease, of such activity (Meyer et al., 1992; Devlin, 1997). A recent study from our research group demonstrated that $HgCl_2$ induced an inhibition on serum ALT activity *in vitro* (unpublished data). It has been suggested that the chemical modification of sulfhydryl group of cysteine is involved in the inactivation of ALT activity (Vedavathi et al., 2004). In fact, Hg is a typical reagent of sulfhydryl groups and its large affinity with these groups contributes to its toxicity (Clarkson, 1997). Other studies demonstrated an increment in ALT activity of experimental animals exposed to $HgCl_2$, revealing

hepatotoxicity (El-Demerdash, 2001; Kumar et al., 2005; Sener et al., 2007; Sharma et al., 2007).

The distinctive responses between lactating and non-lactating rats observed in this study (hepatic d-ALA-D and AST activities) could be associated with some of several physiological changes that occur during lactation (Hanwell and Linzell, 1973; Suzuki et al., 1993). Indeed, physiological changes that occurring during this period, such as increase in blood and plasma volume, decrease in total plasma protein and increases in cardiac output and blood flow, could affect pharmacokinetic of any chemicals, including mercury (Hallén et al., 1996; Houpert et al., 1997, Sundberg et al., 1998). It was previously demonstrated that during lactation the biological half-time of inorganic mercury decreases by 50% due to a higher rate of excretion (i.e., 3.5 days in lactating rats compared to approximately 7 days in non-lactating adult female rats) (Prester et al., 1994; 1997).

The sensitivity of developing animals to various compounds (including metals) may differ from the observed in adults (Jugo, 1976; Kostial et al., 1978; Walsh, 1982; Webb and Holt, 1982; Pereira et al., 1999). Mammals present a high sensitivity to external insults when these are applied in early phases of life. In rodents, this phase happens from birth to weaning (Gottlieb et al., 1977) and is associated with intense development. The breast milk is nutritionally beneficial to the neonate; however, it may also be a deposit for contaminants of the environment. Based on described above, we evaluated the effects of Hg and Zn on neonates exposed to them via breast milk. In fact, the transport of inorganic Hg into milk was previously demonstrated in rats (Sundberg et al., 1991) and in guinea pigs (Yoshida et al., 1994). In the present study, an increase in Hg levels was observed in liver of suckling pups from both groups exposed to $HgCl_2$. In accordance, Sundberg et al. (1991) demonstrated that Hg concentration in milk was linearly correlated to the levels in liver of suckling pups after exposure to Hg via milk. Interestingly, our results showed that the increase in Hg levels observed in the liver of pups seems to be insufficient to cause alterations on biochemical parameters related to hepatic damage. Indeed, Hg hepatic levels in suckling pups exposed to Hg via milk were lower than encountered in maternal livers. In contrast, when young rats were directly exposed to $HgCl_2$ (at the same dose and developmental period) an increase in liver Hg levels (Peixoto et al., 2003; 2007a; 2007b) followed by alterations in hepatic

functions and glycemia (Peixoto and Pereira, 2007) and inhibition of hepatic d-ALA-D activity (Rocha et al., 1995; Peixoto et al., 2003; 2007a) was observed.

The levels of zinc, an essential trace element which is critical to normal growth and development (Krebs, 1999), also were measured in liver of suckling pups from lactating dams exposed to metals during lactation. In accordance with previous studies, it was demonstrated that zinc could be transferred to the pups through maternal milk (Krebs et al., 1985; Karra et al., 1988; Moser-Veillon, 1995). In fact, Zn levels increased about 2-fold in liver of pups from both groups of lactating rats exposed to $ZnCl_2$ (Zn–Sal and Zn–Hg). It is known that Zn is an essential metal to d-ALA-D activity (Tsukamoto et al., 1979; Bevan et al., 1980) and there are a number of studies showing that Zn activates this enzyme (Despaux et al., 1977; Thompson et al., 1977; Nelson, 1981; Bernard and Lauwerys, 1987). In this study, however, the increase of Zn levels in liver of pups was unable to induce any changes in d-ALA-D activity.

In summary, this study demonstrated that lactating rats presented distinct biochemical responses comparing to non-lactating rats exposed to $HgCl_2$ when hepatic parameters were evaluated. Of particular importance, $HgCl_2$-exposure induced an increase in hepatic d-ALA-D activity of lactating rats, but not of non-lactating. Furthermore, Hg-intoxication of dams resulted in an increase in liver Hg levels without affecting any hepatic parameter evaluated in pups.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements:

Financial support-provided by FINEP research grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net)’’ #01.06.0842-00. M.E.P. (307814/2008-4) and A.M.F. (142297/2006-2) are recipients of CNPq fellowships; C.F. is recipient of CAPES fellowships.

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Legends

Figure 1. Hepatic d-ALA-D activity of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 6–7). Bars labeled with different letters are significantly different when considered the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 2. Mercury (a) and zinc (b) levels in liver of lactating and non-lactating rats exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 3–5). Bars labeled with different letters are significantly different when considered the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 3. Mercury (a) and zinc (b) levels in liver of pups from dams exposed to $ZnCl_2$ (27 mg/kg) or saline for five consecutive days and exposed to $HgCl_2$ (5 mg/kg) or saline on the five subsequent days. Data are expressed as mean ± S.E.M. (n = 3–5). Bars labeled with different letters are significantly different when considered the same group of rats (lactating or non-lactating) ($P<0.05$, Duncan's multiple range test).

Figure 4. Histophatological evaluation in hepatic tissue of lactating rats. (a) Lactating rats from Sal–Sal group presented normal histology (H&E 100x). Normal hepatocytes are showed in detail (H&E 400x). (b) Lactating rats from Zn–Hg group showed alterations including loss of lobular architecture and dilated sinusoids (arrows) (H&E 100x). Enlarged, swollen and finely vacuolated hepatocytes are showed in detail (H&E 400x). CV: centrolobular vein.

Tables

Table 1. Absolute and relative liver weights of lactating and non-lactating rats exposed to $ZnCl_2$ and $HgCl_2$

Liver weights	**Sal–Sal**	**Sal–Hg**	**Zn–Sal**	**Zn–Hg**
Lactating				
Absolute (g)	15.40 ± 0.28^{ab}	13.53 ± 0.87^{b}	16.68 ± 0.26^{a}	13.91 ± 0.76^{b}
Relative (%)	4.71 ± 0.11	4.84 ± 0.19	4.93 ± 0.14	4.89 ± 0.14
Non-lactating				
Absolute (g)	9.64 ± 0.41	8.80 ± 0.51	9.67 ± 0.19	8.84 ± 0.44
Relative (%)	4.13 ± 0.18	4.42 ± 0.27	4.15 ± 0.13	4.36 ± 0.13

Data are expressed as mean ± S.E.M. (n = 6–7) and the values followed by different letters in the same line are statistically different ($P<0.05$).

Table 2. Biochemical determinations in plasma from lactating and non-lactating rats exposed to $ZnCl_2$ and $HgCl_2$

Parameters	**Sal–Sal**	**Sal–Hg**	**Zn–Sal**	**Zn–Hg**
Lactating				
ALT (U/mL)	125.18 ± 13.27[a]	65.58 ± 15.87[b]	133.42 ± 11.28[a]	62.50 ± 9.67[b]
AST (U/mL)	56.67 ± 7.68	66.50 ± 6.47	69.08 ± 11.05	72.30 ± 8.78
LDH (U/L)	449.25 ± 30.03	474.59 ± 77.26	439.22 ± 78.83	501.86 ± 71.35
Glucose (mg/dL)	85.59 ± 14.81	114.14 ± 13.67	94.32 ± 10.32	93.86 ± 9.51
Non-lactating				
ALT (U/mL)	64.00 ± 4.43[a]	30.70 ± 2.96[b]	61.60 ± 3.72[a]	38.60 ± 3.75[b]
AST (U/mL)	76.00 ± 3.77[a]	91.20 ± 5.08[b]	78.40 ± 3.53[a]	81.00 ± 1.80[ab]
LDH (U/L)	505.50 ± 39.20	537.70 ± 39.04	442.02 ± 27.00	420.25 ± 36.84
Glucose (mg/dL)	120.31 ± 8.89	134.96 ± 9.50	119.45 ± 6.57	131.11 ± 6.53

Data are expressed as mean ± S.E.M. (n = 6–7) and the values followed by different letters in the same line are statistically different ($P<0.05$).

Table 3. Absolute and relative liver weights and biochemical determinations in pups from lactating rats exposed to $ZnCl_2$ and $HgCl_2$ during lactation

Parameters	**Sal–Sal**	**Sal–Hg**	**Zn–Sal**	**Zn–Hg**
Absolute liver weight (g)	0.68 ± 0.03[a]	0.50 ± 0.04[b]	0.69 ± 0.03[a]	0.50 ± 0.03[b]
Relative liver weight (%)	2.70 ± 0.10	2.58 ± 0.09	2.74 ± 0.08	2.63 ± 0.06
Hepatic d-ALA-D activity[1]	27.29 ± 0.74	26.61 ± 1.98	29.04 ± 1.76	29.56 ± 1.92
ALT (U/mL)	28.25 ± 2.68	31.66 ± 3.98	26.50 ± 1.42	29.42 ± 2.89
AST (U/mL)	40.33 ± 3.00	47.42 ± 7.15	43.58 ± 8.40	40.50 ± 3.04
LDH (U/L)	318.38 ± 22.76	348.23 ± 39.74	335.80 ± 31.97	430.15 ± 50.49
Glucose (mg/dL)	86.92 ± 5.73	86.04 ± 6.08	98.14 ± 11.04	83.38 ± 11.24

Data are expressed as mean ± S.E.M. (n = 6–7) and the values followed by different letters in the same line are statistically different ($P<0.05$).

[1] Expressed as nmol PBG/ mg protein/ h.

Figures

Figure 1.

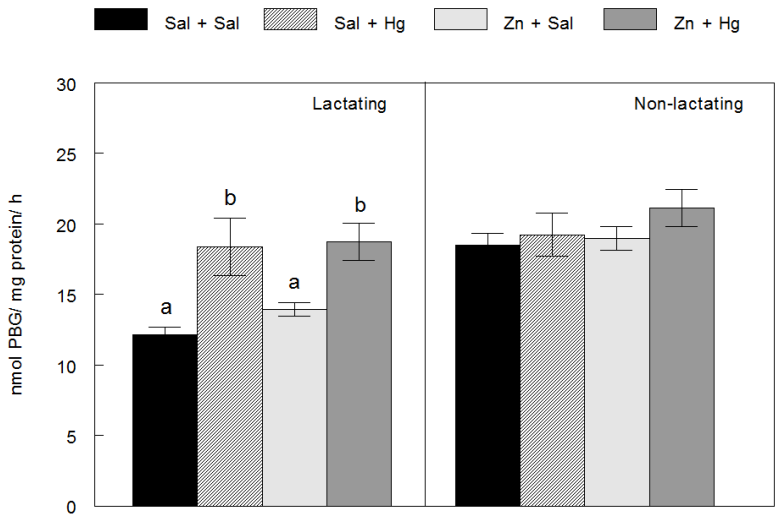


Figure 2.

Figure 2a.

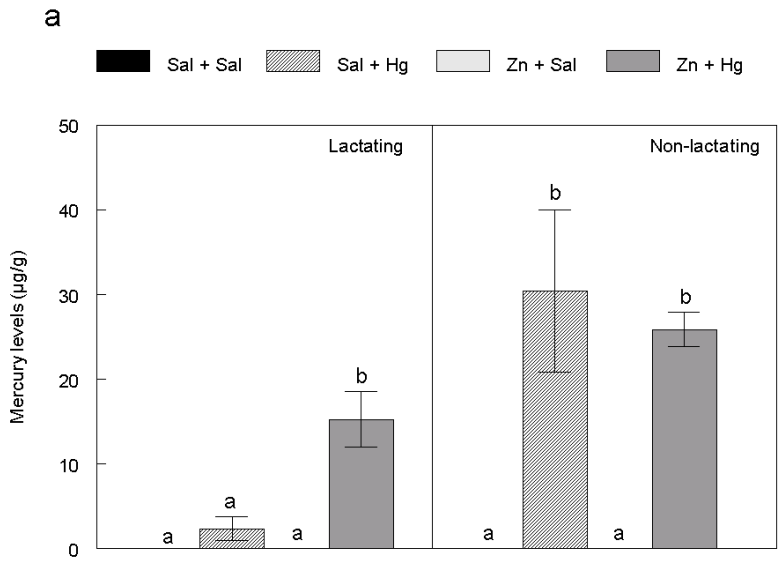


Figure 2b.

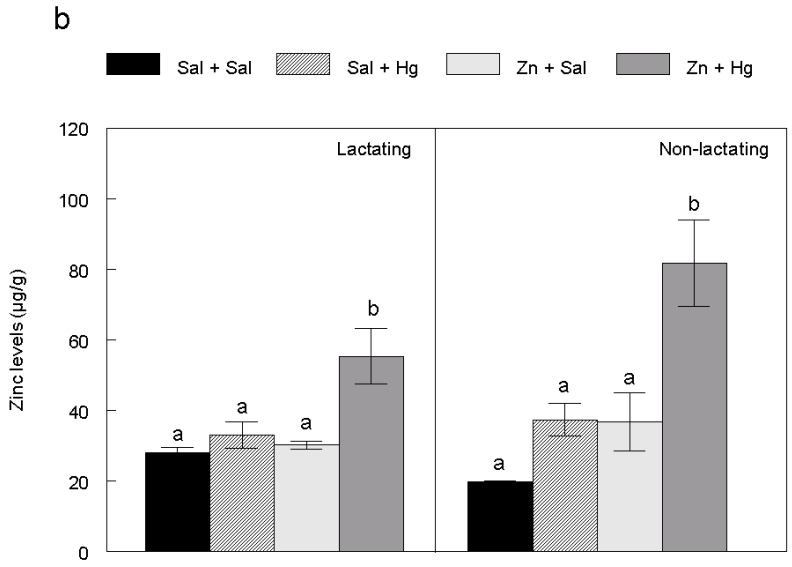


Figure 3.

Figure 3a.

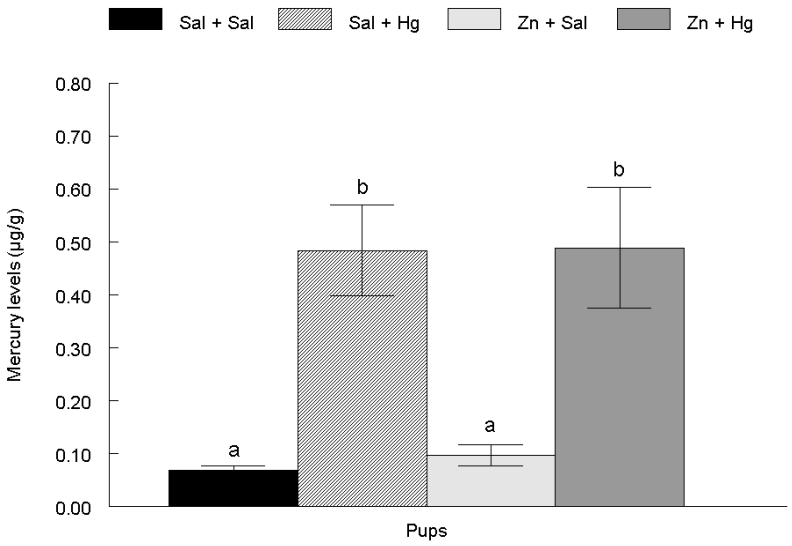


Figure 3b.

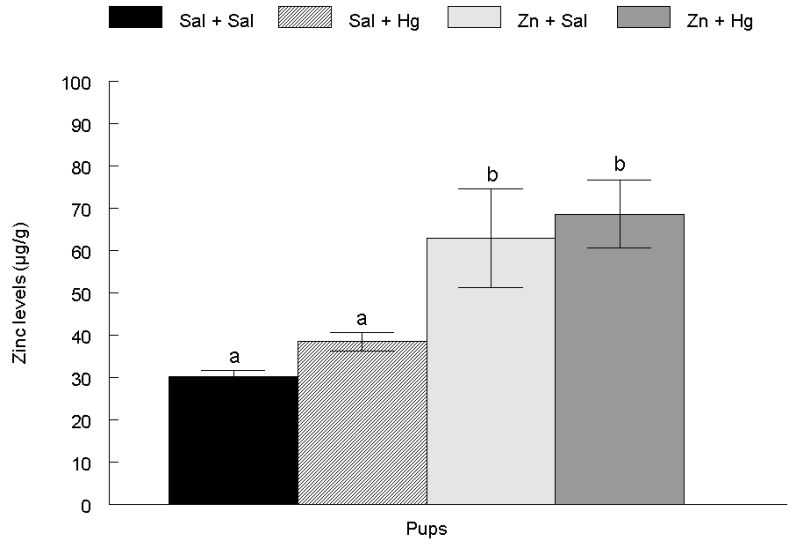
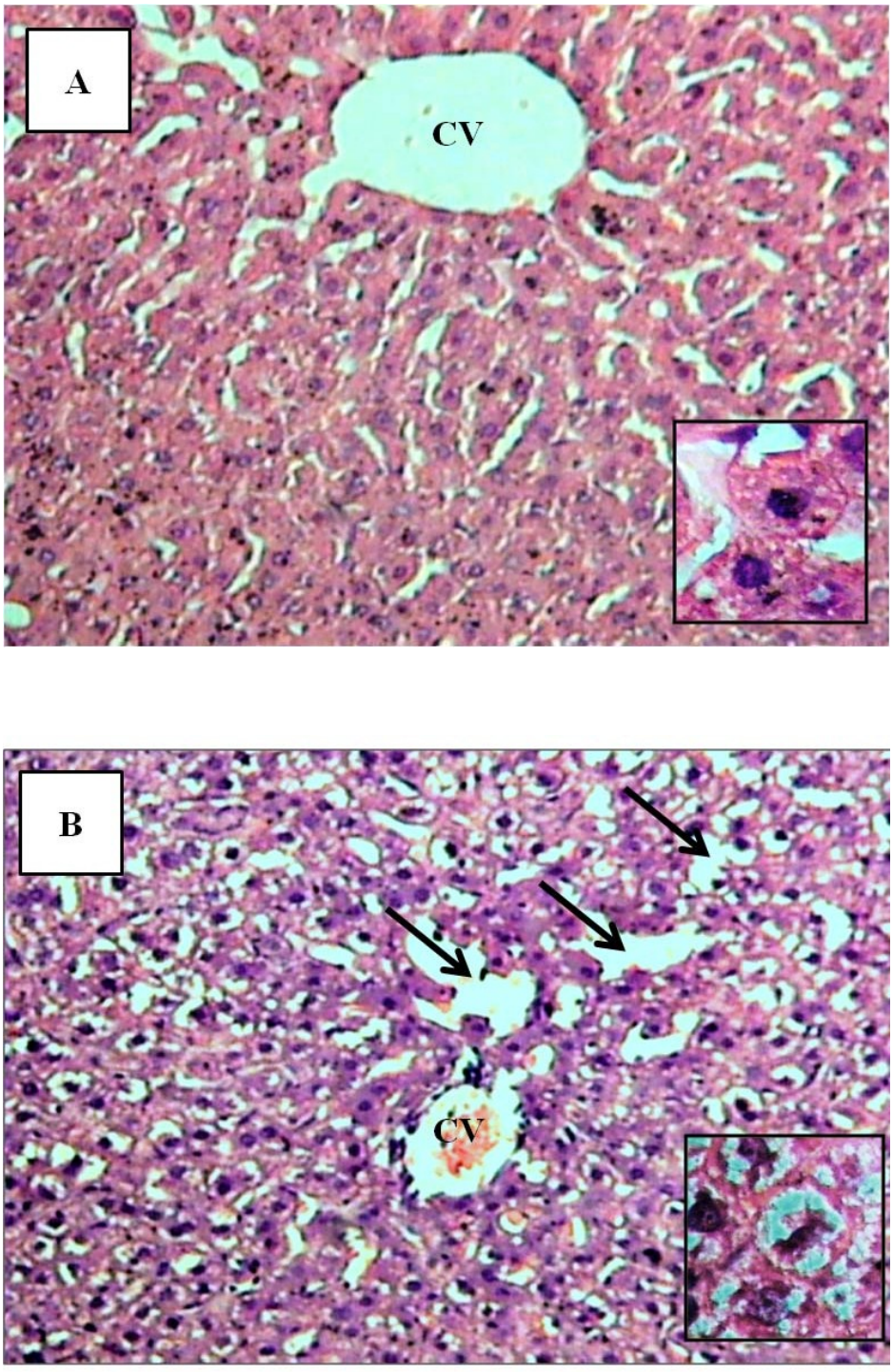


Figure 4.



4. DISCUSSÃO

O mercúrio é um metal não-essencial altamente tóxico, em parte devido a sua característica de ficar retido nos tecidos por um longo tempo tornando possível sua redistribuição subsequente (Goyer, 1996). Tem sido demonstrado que no período de lactação ocorre uma diminuição da meia-vida biológica deste metal (Prester *et al.*, 1994; 1997), o que pode modificar seus efeitos tóxicos durante este período. Embora existam na literatura diversos trabalhos demonstrando os efeitos tóxicos das formas inorgânicas de mercúrio e a distribuição deste metal entre os órgãos, não existem estudos que demonstrem diferença na susceptibilidade aos danos causados pela exposição ao cloreto de mercúrio ($HgCl_2$) no período de lactação. Estudos recentes mostraram que algumas das alterações bioquímicas graves induzidas por este metal são prevenidas pela pré-exposição ao zinco em ratos jovens (Peixoto *et al.*, 2003; Peixoto & Pereira, 2007; Franciscato *et al.*, 2011). Considerando as informações acima descritas, este trabalho teve como objetivo comparar os possíveis efeitos tóxicos da exposição ao $HgCl_2$ sobre parâmetros bioquímicos indicativos de toxicidade renal e hepática em ratas lactantes e não-lactantes e investigar se a pré-exposição ao zinco é capaz de prevenir os efeitos tóxicos causados pelo mercúrio. Além disso, foram avaliados os efeitos dos metais sobre os filhotes expostos a eles através do leite materno.

Os dados apresentados neste trabalho demostram que o efeito do $HgCl_2$ sobre parâmetros bioquímicos indicativos de toxicidade renal e hepática difere entre as ratas lactantes e não-lactantes. No **Manuscrito I**, verificamos que a exposição ao $HgCl_2$ induziu toxicidade renal apenas nas ratas não-lactantes, conforme demonstrado pelo aumento dos níveis plasmáticos de uréia e creatinina, pela inibição na atividade da enzima d-ALA-D renal e pelas alterações histopatológicas nos rins destes animais. Além disso, foi demonstrado que o $ZnCl_2$ preveniu as alterações renais induzidas pelo $HgCl_2$, à exemplo do anteriormente demonstrado em estudos conduzidos com ratos jovens (Peixoto *et al.*, 2003; Peixoto & Pereira, 2007; Franciscato *et al*., 2011), sugerindo, dessa forma, que o $ZnCl_2$ pode servir como uma alternativa promissora no tratamento preventivo de casos de intoxicação por mercúrio. No **Manuscrito II**, no qual foram avaliados os efeitos da exposição ao $HgCl_2$ sobre o tecido hepático, verificamos que o $HgCl_2$ causou um aumento dos níveis de mercúrio hepático e uma diminuição na atividade da enzima ALT plasmática tanto nas ratas lactantes quanto nas não-lactantes.

Por outro lado, a exposição ao $HgCl_2$ induziu um aumento na atividade da enzima d-ALA-D hepática apenas nas ratas lactantes e um aumento na atividade da enzima AST plasmática, um marcador de hepatotoxicidade, apenas nas ratas não-lactantes.

As ratas lactantes e não-lactantes expostas ao $HgCl_2$ apresentaram uma perda de peso corporal que está associada a uma diminuição do consumo de ração. Estes resultados podem estar diretamente relacionados ao efeito anorexigênico deste metal (Freundt & Ibrahim, 1990; Counter & Buchanan, 2004). Ao final do período de exposição aos metais, foram observadas alterações no peso dos órgãos analisados. O peso relativo dos rins, alvos primários da toxicidade das formas inorgânicas do mercúrio (ATSDR, 1994; Zalups, 1993; Zalups & Lash, 1994), estava aumentado nas ratas lactantes (cerca de 55%) e nas não-lactantes (cerca de 95%). O aumento mais pronunciado no peso e no tamanho dos rins das ratas não-lactantes (**Apêndices, Item A**) parece estar relacionado às alterações estruturais causadas pelo mercúrio neste órgão, conforme comprovado pela análise histológica do tecido renal (discutidas a seguir). O peso relativo do fígado das ratas lactantes e não-lactantes, por sua vez, não foi alterado pela exposição ao $HgCl_2$, confirmando que a diminuição observada no peso absoluto deste órgão (não significativa) está diretamente relacionada à diminuição do peso corporal.

A exposição ao $HgCl_2$ causou uma diminuição da atividade da enzima d-ALA-D sanguínea das ratas lactantes e não-lactantes, refletindo os níveis de mercúrio no sangue das ratas (aumentado em ambas) e confirmando estudos prévios que demostraram que esta enzima pode servir como um biomarcador da exposição ao mercúrio (Bernard & Lauwerys, 1987; Oskarsson & Fowler, 1987; Peixoto *et al.*, 2004). Um resultado interessante deste estudo foi que a exposição ao $HgCl_2$ causou uma inibição na atividade da enzima d-ALA-D renal das ratas não-lactantes e não afetou a atividade da enzima das lactantes. Tem sido demonstrado por diversos autores que a d-ALA-D renal é inibida pelo mercúrio inorgânico tanto em ratos jovens (Rocha *et al.*, 1995; Peixoto *et al.*, 2003; 2004; 2007b; Roza *et al.*, 2005; Franciscato *et al.*, 2011) quanto em adultos (Perotoni *et al*., 2004a; 2004b; Emanuelli *et al.*, 1996; Augusti *et al.*, 2007; 2008). É importante observar que, nas ratas não-lactantes, a atividade da enzima d-ALA-D sanguínea foi inibida em concentrações de mercúrio bem menores do que aquelas encontradas nos rins. Este resultado está de acordo com dados da literatura que demonstraram que a enzima d-ALA-D sanguínea é mais sensível aos efeitos do mercúrio do que a de origem

renal (Rocha *et al.*, 1995). Portanto, apesar da diferença observada no período de lactação em relação ao efeito do mercúrio sobre a atividade da enzima d-ALA-D renal, a enzima sanguínea pode ser considerada um bom indicador de exposição ao mercúrio também para as lactantes.

Por outro lado, a atividade da enzima d-ALA-D apresentou-se aumentada apenas no fígado das ratas lactantes expostas ao $HgCl_2$. Este efeito do mercúrio sobre a atividade desta enzima foi inesperado. Diversos estudos têm demonstrado que a d-ALA-D hepática é sensível à ação inibitória do mercúrio inorgânico tanto *in vivo* (Rocha *et al.*, 1995; Emanuelli *et al.*, 1996; Peixoto *et al.*, 2003; 2007b; Franciscato *et al.*, 2011), quanto *in vitro* (Rocha *et al.*, 1995; Peixoto *et al.*, 2004). Em um estudo recente realizado em cultura primária de hepatócitos de *H. malabaricus*, um peixe selvagem coletado na bacia Amazônica, o metilmercúrio, uma forma orgânica de mercúrio, induziu um aumento na atividade da d-ALA-D (cerca de 48%) (Filipak Neto *et al.*, 2008). Não sabemos ao certo qual foi o motivo para o aumento observado na atividade da enzima das ratas lactantes. Entretanto, independentemente do mecanismo pelo qual o $HgCl_2$ induziu esse aumento, os dados apresentados demonstram claramente que o comportamento da enzima d-ALA-D hepática de ratas lactantes é distinto daquele observado nas ratas não-lactantes.

O $HgCl_2$ é reconhecido como um agente nefrotóxico que induz alterações na função renal (ATSDR, 1994; Zalups & Lash, 1994). Os rins são os órgãos primários que acumulam mercúrio inorgânico e a presença deste metal no interior das células tubulares pode ser vista dentro de algumas horas após a exposição ao mercúrio (Zalups, 1993). No presente estudo, foi observado um aumento nos níveis de mercúrio nos rins das ratas lactantes, bem como das não-lactantes expostas ao $HgCl_2$. A exposição prévia ao $ZnCl_2$ não impediu o acúmulo de mercúrio renal nas ratas não-lactantes e potencializou este efeito nas lactantes. Apesar do aumento nos níveis de mercúrio renal causado pela exposição prévia ao $ZnCl_2$ observado nas ratas lactantes, o metal não induziu qualquer alteração nos parâmetros bioquímicos indicativos de toxicidade renal avaliados neste estudo. O efeito do $ZnCl_2$ sobre o aumento da concentração de mercúrio nos rins de animais pré-expostos a ele, e posteriormente expostos ao $HgCl_2$, foi previamente observado em trabalhos do nosso grupo de pesquisa (Peixoto *et al.*, 2003; 2007a; Franciscato *et al.*, 2011). De fato, os parâmetros bioquímicos indicativos da nefrotoxicidade induzida pelo $HgCl_2$ foram gravemente alterados apenas nas ratas não-

lactantes. Conforme discutido anteriormente, foi observado um aumento no peso renal tanto das ratas lactantes quanto das não-lactantes expostas ao $HgCl_2$. Embora tenha sido relatado que o aumento no peso dos rins é um indicador de nefrotoxicidade (Sharratt & Frazer, 1963; Kluwe *et al.*, 1981; Simmons, *et al.*, 1995), alguns estudos demonstraram que apenas uma lesão renal grave (que resulta em redução de 50 a 75% na taxa de filtração glomerular) pode causar aumento dos níveis plasmáticos de uréia e creatinina (Goldstein & Schnellmann, 1996; Dinour & Brezis, 1997). No presente estudo, as ratas não-lactantes expostas ao $HgCl_2$ apresentaram um aumento do peso renal acompanhado de um aumento pronunciado dos níveis plasmáticos de uréia e creatinina, indicando uma lesão renal grave nestes animais. A avaliação histopatológica corroborou com esses achados, uma vez que apenas as ratas não-lactantes expostas ao $HgCl_2$ apresentaram alterações histológicas no tecido renal, incluindo dilatação tubular, achatamento das células epiteliais com material proteináceo preenchendo o lúmen dos túbulos (um indicador de dano tubular extenso) e fibrose intersticial (caracterizada por aumento da deposição de colágeno). Já foi demonstrado que a fibrose está correlacionada com a perda da função renal (Cohen, 1995). É importante mencionar que uma alta taxa de mortalidade, aproximadamente 32%, foi observada no grupo de ratas não-lactantes expostas ao $HgCl_2$, enquanto que no grupo de ratas não-lactantes pré-expostas ao $ZnCl_2$ essa taxa foi de apenas 5%. Em conformidade, Barnes *et al.* (1980) demonstraram que os íons Hg^{2+} concentram-se nos rins após a exposição ao $HgCl_2$, produzindo danos ao sistema tubular renal, podendo levar à morte por uremia.

Neste estudo, as ratas lactantes e não-lactantes apresentaram um aumento nos níveis de mercúrio hepático após a exposição ao $HgCl_2$. Além disso, foi demonstrado pela primeira vez que a exposição prévia ao $ZnCl_2$ causa um maior acúmulo de mercúrio no fígado de ratas lactantes. A avaliação histopatológica corroborou com os resultados da análise de mercúrio no fígado das lactantes, uma vez que apenas as ratas lactantes do grupo exposto a ambos os metais (Zn e Hg) apresentaram alterações histológicas no tecido hepático, incluindo perda da arquitetura lobular, degeneração hepatocelular (hepatócitos alargados, inchados e finamente vacuolizados) e dilatação dos sinusóides. Estudos prévios demonstraram que a exposição ao $HgCl_2$ induz diversas alterações histopatológicas no fígado de animais experimentais incluindo necrose centrolobular, cariólise, cariorrexia e vacuolização citoplasmática e pronunciados espaços entre os sinusóides (Kumar *et al.*, 2005; Sharma *et al.*, 2000; 2002; 2007).

Apesar de terem sido detectadas alterações histológicas no tecido hepático das ratas lactantes expostas a ambos os metais (Zn-Hg), não foram observadas diferenças nos parâmetros bioquímicos relacionadas à exposição ao mercúrio. As ratas lactantes de ambos os grupos expostos ao $HgCl_2$ (Sal-Hg e Zn-Hg) apresentaram um aumento similar na atividade da enzima d-ALA-D hepática (cerca de 40%), o qual não foi prevenido pela pré-exposição ao $ZnCl_2$. O efeito do $HgCl_2$ sobre a atividade da enzima d-ALA-D hepática das lactantes também não pode ser explicado pelos níveis de mercúrio presentes neste tecido, uma vez que os níveis deste metal foram maiores no fígado das ratas lactantes expostas a ambos os metais (Zn-Hg). Além disso, a atividade da d-ALA-D das ratas não-lactantes de ambos os grupos expostos ao $HgCl_2$ não foi alterada, apesar da elevação dos níveis de mercúrio hepático. Em ratos jovens intoxicados por mercúrio, níveis elevados de mercúrio no tecido hepático foram encontrados 24 horas após o final da exposição ao $HgCl_2$, sendo que estes animais apresentaram inibição na atividade da enzima d-ALA-D hepática (Peixoto *et al.*, 2003).

As enzimas AST, ALT e LDH são indicadores sensíveis de dano hepatocelular (Meyer *et al.*, 1992; Devlin, 1997). No presente estudo, a atividade da LDH não foi alterada pela exposição ao $HgCl_2$ em ambas as ratas lactantes e não-lactantes. Em contrapartida, foi observado um incremento de 20% na atividade da AST plasmática de ratas não-lactantes 24 horas após o término do período de exposição ao Hg e este efeito foi parcialmente prevenido quando os animais foram pré-expostos a $ZnCl_2$, indicando que a pré-exposição ao zinco pode ser uma alternativa no tratamento preventivo contra a hepatotoxicidade induzida pelo mercúrio. Aumentos na atividade da AST relacionado à exposição $HgCl_2$ foram demonstrados previamente por diversos autores (El-Demerdash, 2001; Kumar *et al.*, 2005; Sener *et al.*, 2007; Sharma *et al.*, 2007). Por outro lado, observou-se uma diminuição na atividade plasmática da ALT de ratas lactantes (cerca de 48%) e não-lactantes (cerca de 52%) expostas ao $HgCl_2$ e este efeito não foi prevenido pela pré-exposição ao $ZnCl_2$. Em conformidade com os resultados deste estudo, Peixoto & Pereira (2007) demonstraram uma redução de 40% na atividade da ALT de ratos jovens expostos ao $HgCl_2$. Em um estudo recente foi demonstrado que o efeito do mercúrio na redução da atividade da ALT persiste por um longo período após o término da intoxicação pelo mercúrio (Franciscato *et al.*, 2011). Nesse estudo, os ratos mais sensíveis à exposição ao mercúrio apresentaram uma diminuição de 66,2% na atividade da ALT sérica 21 dias após o término do período de exposição. O efeito do

mercúrio sobre a atividade da ALT observado neste estudo não indica lesão hepática, pois em uma lesão dos hepatócitos ocorre o extravasamento desta enzima com o consequente aumento dos seus níveis no sangue (Meyer *et al.*, 1992; Devlin, 1997). Um estudo do nosso grupo de pesquisa demonstrou que o $HgCl_2$ inibe a atividade da ALT sérica *in vitro* (dados submetidos à publicação). Tem sido sugerido que a modificação química dos grupos sulfidrílicos da cisteína está envolvida na inativação da atividade da ALT (Vedavathi *et al.*, 2004). De fato, o mercúrio possui alta afinidade por grupos sulfidrílicos o que contribui para a toxicidade deste metal (Clarkson, 1997). Outros estudos mostraram um aumento na atividade da ALT em animais experimentais expostos ao $HgCl_2$, revelando que este metal pode induzir toxicidade hepática (El-Demerdash, 2001; Kumar *et al.*, 2005; Sener *et al.*, 2007; Sharma *et al.*, 2007) dependendo do modelo de exposição.

As diferentes respostas observadas entre as ratas lactantes e não-lactantes em relação à toxicidade do $HgCl_2$ (na atividade da enzima d-ALA-D renal e hepática; na atividade da AST plasmática; na histologia dos tecidos renal e hepático; na mortalidade, entre outros) podem estar associadas com algumas das diversas alterações fisiológicas que ocorrem durante a lactação (Hanwell & Linzell, 1973; Suzuki *et al.*, 1993). Sabe-se que as mudanças fisiológicas que ocorrem durante este período, como o aumento do volume de sangue e plasma, a diminuição da concentração de proteína plasmática total, o aumento do débito cardíaco e o aumento do fluxo sanguíneo a certos órgãos (glândulas mamárias, fígado e trato gastrointestinal), alguns dos quais envolvidos diretamente no processo de lactação, podem afetar a farmacocinética de diversos agentes químicos, incluindo o mercúrio (Hallén *et al.*, 1996; Houpert *et al.*, 1997; Sundberg *et al.*, 1998). De fato, foi mostrado previamente que a meia-vida biológica do mercúrio inorgânico diminui em 50% durante a lactação devido a uma maior taxa de excreção do metal (de cerca de 7 dias em ratas adultas não-lactantes para apenas 3,5 dias em ratas lactantes) (Prester *et al.*, 1994; 1997). Além disso, um importante mecanismo que pode ajudar a explicar, pelo menos em parte, a redução da toxicidade do mercúrio em ratas lactantes é o aumento da síntese de metalotioneína (MT) durante este período (Solaiman *et al.*, 2001). As MT são proteínas que possuem uma alta afinidade por metais, principalmente os divalentes (Chan *et al.*, 2002; Dabrio *et al.*, 2002). Essas proteínas parecem estar envolvidas no controle homeostático do metabolismo de metais, na adaptação celular ao estresse e podem ser modificadas pela exposição a diversos

agentes, tais como metais, compostos químicos, radiações ionizantes e hormônios (Dunn *et al.*, 1987; Shimada *et al.*, 1997). Sabe-se que durante o período intermediário da lactação ocorre um aumento na síntese de MT, o que sugere que essa proteína pode estar envolvida no metabolismo dos metais essenciais requeridos para o desenvolvimento dos neonatos num período em que há uma demanda aumentada por nutrientes (Solaiman *et al.*, 2001). No presente estudo, as ratas lactantes foram expostas ao $HgCl_2$ do 8º ao 12º dia de lactação, próximo ao período reconhecido como sendo o período de pico da síntese de MT em roedores nos órgãos estudados (Solaiman *et al.*, 2001). Devido ao alto teor de cisteína na molécula de MT, é possível que esta proteína sequestre uma alta porcentagem deste metal tóxico em um complexo inerte, tornando-o indisponível para interagir com as organelas ou sistemas enzimáticos sensíveis à exposição ao mercúrio (Klaassen *et al.*, 1999; Nath *et al.*, 2000; Romero-Isart & Vasak, 2002; Peixoto *et al.*, 2003). Do ponto de vista toxicológico, a proteção observada em ratas lactantes contra a toxicidade causada pelo $HgCl_2$ é muito importante uma vez que o desenvolvimento dos neonatos é altamente dependente da saúde de sua mãe durante esse período.

A pré-exposição ao zinco preveniu a inibição da atividade da enzima d-ALA-D renal e a nefrotoxicidade induzida pelo mercúrio inorgânico em ratas não-lactantes. A prevenção da inibição da atividade da d-ALA-D renal pode ser explicada pelo fato do zinco ser requerido para a manutenção dos grupos –SH da enzima no estado reduzido, tendo assim um papel crítico na atividade desta (Tsukamoto *et al.*, 1979; Beber *et al.*, 1998; Emanuelli *et al.*, 1998). Os efeitos preventivos do $ZnCl_2$ contra a nefrotoxicidade induzida pelo $HgCl_2$ têm sido associados com a sua capacidade de induzir a síntese de proteínas de desintoxicação, como as MT e outras proteínas ligantes de metais (Dunn *et al.*, 1987; Zalups & Cherian, 1992; Kondoh *et al.*, 2003; Peixoto *et al.*, 2003; 2007a; Szczurek, *et al.*, 2009). Estudos realizados pelo nosso grupo mostraram previamente que a pré-exposição ao $ZnCl_2$ previne o dano renal e o efeito inibitório sobre a atividade da enzima d-ALA-D renal induzida por $HgCl_2$ em ratos jovens, concomitantemente ao aumento do conteúdo de MT (Peixoto *et al.*, 2003; 2007a). Além disso, outros autores também demonstraram que o dano renal causado pelo mercúrio inorgânico pode ser prevenido pela pré-indução de MT renal (Webb & Magos, 1976; Zalups & Cherian, 1992). Baseado nisto, sugere-se que um aumento nos níveis de MT renal induzido pelo

$ZnCl_2$ pode ser um dos mecanismos responsáveis, pelo menos em parte, pela redução da toxicidade do mercúrio em ratas não-lactantes.

No presente estudo foi demonstrado que a exposição ao $HgCl_2$ causa uma diminuição dos níveis sanguíneos de zinco em ratas lactantes e não-lactantes e que a pré-exposição ao $ZnCl_2$ foi capaz de prevenir os efeitos do mercúrio sobre esse parâmetro apenas no grupo de ratas não-lactantes. Não foram observadas alterações nos níveis renais e hepáticos de zinco nas ratas lactantes e nas não-lactantes quando essas foram expostas exclusivamente ao $HgCl_2$. Por outro lado, um aumento nos níveis de zinco foi detectado nesses tecidos quando as ratas (lactantes e não-lactantes) foram pré-expostas ao $ZnCl_2$ e depois ao $HgCl_2$. Nos rins esse aumento parece estar diretamente relacionado com a exposição ao $ZnCl_2$, uma vez que um aumento similar foi observado no grupo de ratas expostas exclusivamente ao $ZnCl_2$. Já nas ratas não-lactantes o aumento foi observado apenas no grupo exposto a ambos os metais, o que sugere que o mercúrio presente no tecido hepático pode estar causando alguma alteração na homeostasia do zinco nesse tecido.

A sensibilidade dos animais em desenvolvimento a diversos agentes tóxicos (incluindo os metais) pode diferir daquela observada em adultos (Jugo, 1976; Kostial *et al.*, 1978; Walsh, 1982; Webb & Holt, 1982; Pereira *et al.*, 1999). Os mamíferos apresentam alta sensibilidade a insultos externos quando estes são aplicados em fases precoces do desenvolvimento, como o período neonatal. Nos roedores, essa fase tem início ao nascimento e estende-se até o desmame (Gottlieb *et al.*, 1977) e está associada com um intenso desenvolvimento dos órgãos. Durante esse período os neonatos obtêm os nutrientes necessários para o desenvolvimento inicial através do leite materno. O leite materno é o alimento ideal para os neonatos em função de sua digestibilidade, composição química balanceada e capacidade de gerar imunidade, no entanto, também pode servir como um depósito para contaminantes presentes no ambiente (Gartner, *et al*., 1997). Baseado no descrito acima, os efeitos do mercúrio e do zinco nos neonatos expostos a eles via leite materno também foram avaliados. O transporte de mercúrio inorgânico no leite materno foi previamente demonstrado em ratos (Sundberg *et al.*, 1991b) e em porcos da Índia (Yoshida *et al.*, 1994). No presente estudo, foi observado um aumento nos níveis de mercúrio nos rins e no fígado dos filhotes de ambos os grupos expostos ao $HgCl_2$. Os resultados deste estudo estão de acordo com os dados de Sundberg *et al.* (1991b) que mostraram que a concentração de mercúrio no leite está

linearmente correlacionada com os níveis deste metal nos rins e no fígado dos lactentes após a exposição ao mercúrio através do leite materno. É interessante notar que o aumento nos níveis de mercúrio observados nos rins e no fígado de filhotes neste estudo foi insuficiente para causar alterações nos parâmetros bioquímicos indicativos de danos nestes tecidos. De fato, os níveis de mercúrio detectados nos rins dos filhotes expostos ao mercúrio através do leite materno são muito baixos (de 0,90 a 1,30%) se comparados aos níveis deste metal detectados nos rins das lactantes. Além disso, os níveis de mercúrio detectados no fígado dos lactentes expostos ao mercúrio através do leite materno também foram inferiores aos encontrados no fígado materno. A única alteração fisiológica importante observada nos filhotes foi o menor ganho de peso corporal durante o período de exposição ao mercúrio, que pode ser um reflexo do estado nutricional das lactantes, as quais consumiram menos ração e apresentaram perda de peso corporal. Ao contrário, quando ratos jovens foram expostos diretamente ao $HgCl_2$ (na mesma dose administrada nas lactantes e no mesmo período de desenvolvimento dos filhotes) foi observado um aumento nos níveis deste metal nos tecidos renal e hepático (Peixoto *et al.*, 2003; 2007a; 2007b) seguido por alterações nas funções destes tecidos, na glicemia (Peixoto & Pereira, 2007) e pela inibição da atividade da enzima d-ALA-D renal e hepática (Rocha *et al.*, 1995; Peixoto *et al.*, 2003; 2007b).

Os níveis de zinco, um elemento essencial requerido para o crescimento e desenvolvimento (Krebs, 1999), também foram medidos no sangue, nos rins e no fígado dos filhotes de ratas lactantes expostas aos metais durante a lactação. Os filhotes das ratas lactantes expostas ao $HgCl_2$ apresentaram níveis renais de zinco semelhantes aos dos filhotes das ratas controle. Portanto, a diminuição dos níveis sanguíneos de zinco materno não interferiu na disponibilização deste metal essencial para os seus filhotes. Estes dados estão de acordo com dados da literatura que demonstraram que a concentração de zinco no leite materno dificilmente é afetada por uma baixa ingestão ou por uma diminuição dos níveis desse elemento (King, 2000; Sian *et al.*, 2002). Dessa forma, é garantido aos neonatos, alimentados exclusivamente via leite materno, a quantidade adequada de zinco (de Ferrer *et al.*, 2001). Além disso, foi observado um aumento nos níveis de zinco (cerca de duas vezes) no fígado dos filhotes das ratas previamente expostas ao $ZnCl_2$ e posteriormente à solução salina ou $HgCl_2$ (grupos Zn-Sal e Zn-Hg). Este resultado indica que, independentemente dos efeitos da exposição ao mercúrio, a exposição materna ao $ZnCl_2$ aumentou a disponibilidade desse metal para os

filhotes. Esses dados estão de acordo com estudos prévios da literatura que demonstraram que os níveis de zinco no leite materno são influenciados pela suplementação materna com zinco (Krebs *et al.*, 1985; Karra *et al.*, 1988). Sabe-se que o zinco é um metal essencial para a atividade da d-ALA-D (Tsukamoto *et al.*, 1979; Bevan *et al.*, 1980) e há uma série de estudos que demostraram que o zinco pode ativar esta enzima (Despaux *et al.*, 1977, Thompson *et al.*, 1977; Nelson, 1981; Bernard & Lauwerys, 1987). Neste estudo, entretanto, o aumento dos níveis de zinco no fígado dos filhotes não induziu alterações na atividade da d-ALA-D neste tecido.

Por fim, este estudo demonstrou que as ratas lactantes apresentam respostas bioquímicas diferentes em comparação as ratas não-lactantes expostas ao $HgCl_2$ quando parâmetros indicativos de toxicidade renal e hepática foram avaliados. Sugere-se que isso pode estar relacionado às alterações fisiológicas que ocorrem durante o período de lactação. Portanto, estudos adicionais são necessários para elucidar a causa da diferença de sensibilidade das ratas lactantes e não-lactantes ao mercúrio inorgânico apresentada pelos animais neste trabalho.

5. CONCLUSÕES

Observando os resultados descritos, podemos concluir que:

1) A exposição ao cloreto de mercúrio:

a) induz perda de peso corporal, aumento no peso renal e diminuição no consumo de ração em ratas lactantes e não-lactantes.

b) aumenta a deposição de mercúrio em sangue, rins e fígado de ratas lactantes e não-lactantes.

c) induz alterações na atividade da enzima d-ALA-D, cuja quantificação serve para o biomonitoramento da exposição a metais divalentes:

- inibe a atividade da enzima sanguínea, em ratas lactantes e não-lactantes;

- inibe a atividade da enzima renal em ratas não-lactantes; e

- aumenta a atividade da enzima hepática em ratas lactantes.

d) prejudica a função renal das ratas não-lactantes, uma vez que os níveis plasmáticos de uréia e creatinina encontram-se elevados.

e) induz alterações histológicas no tecido renal das ratas não-lactantes.

2) A exposição ao cloreto de zinco durante os cinco dias que precedem a exposição ao mercúrio:

a) preveniu os efeitos deletérios causados pelo mercúrio em ratas não-lactantes pois, apesar de não prevenir o acúmulo de mercúrio no tecido renal, tornou-o indisponível para causar toxicidade, conforme evidenciado pela prevenção das alterações bioquímicas, da insuficiência renal e da mortalidade dos animais;

b) o zinco deve ser empregado com cautela no período de lactação pois a pré-exposição a este metal resultou num maior acúmulo de mercúrio nos rins e no fígado e induziu alterações histológicas no tecido hepático das ratas lactantes expostas ao mercúrio.

3) A exposição ao mercúrio via leite materno afeta o desenvolvimento e crescimento normal dos lactentes, conforme verificado pelo prejuízo no ganho de peso corporal.

E, por fim, as ratas adultas não-lactantes mostraram-se mais sensíveis aos efeitos tóxicos do cloreto de mercúrio no protocolo de exposição empregado.

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APÊNDICES

A. Aspecto macroscópico dos rins de ratas adultas não-lactantes

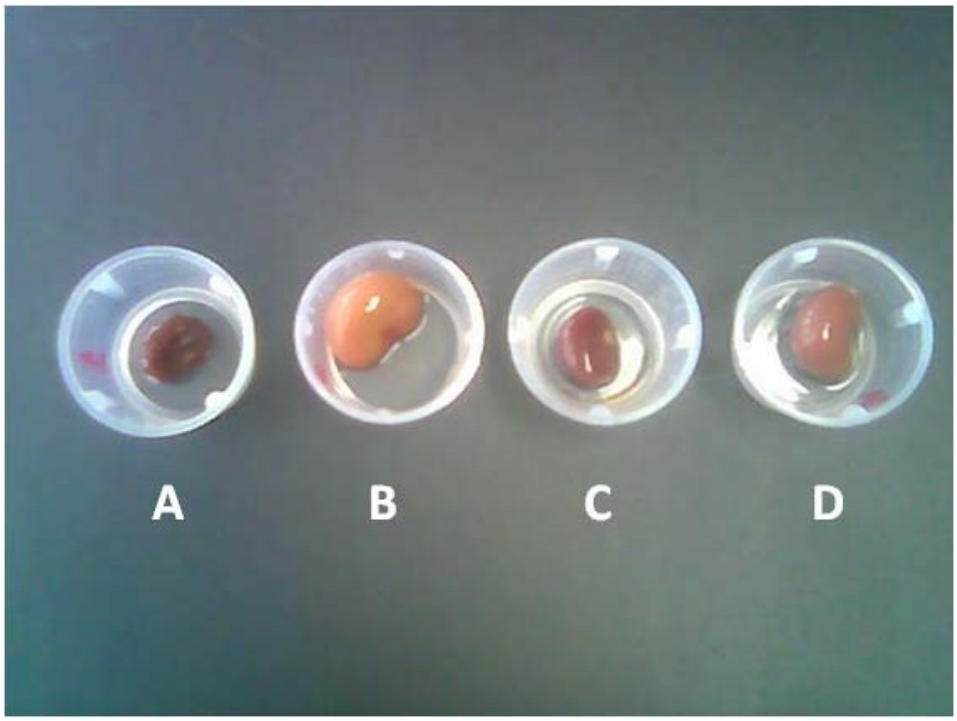

Figura 1. Imagem dos rins de ratas não-lactantes: (A) Grupo Sal–Sal; (B) Grupo Sal–Hg; (C) Grupo Zn–Sal; (D) Grupo Zn–Hg.

B. Manuscrito III

Mercuric chloride exposure during lactational period increases mercury levels in cerebrum and cerebellum of lactating rats without affecting acetylcholinesterase activity

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Manuscrito submetido ao periódico *Neuroscience Letters*

Elsevier Editorial System(tm) for Neuroscience Letters
Manuscript Draft

Manuscript Number:

Title: Mercuric chloride exposure during lactational period increases mercury levels in cerebrum and cerebellum of lactating rats without affecting acetylcholinesterase activity

Article Type: Research Paper

Keywords: mercury; acetylcholinesterase; cerebrum; cerebellum; lactation; pups

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Abstract: The objective of this study was to investigate the effects of maternal exposure to mercuric chloride (HgCl2) during mid-lactation period on acetylcholinesterase (AChE) activity from cerebrum and cerebellum of lactating rats (dams) and their pups. Dams were subcutaneously exposed to saline or HgCl2 (5 mg/kg) from 8th to 12th day of lactation. HgCl2 exposure induced a significant loss of body weight in dams and a lower body weight gain in their pups (indirectly exposed to Hg via maternal milk) without change absolute cerebrum and cerebellum weights. On the other hand, cerebrum-to-body weight ratio was significantly increased in dams and pups exposed to HgCl2 whereas cerebellum-to-body weight ratio was significantly increased only in dams exposed to HgCl2. AChE activity from cerebrum and cerebellum of dams and pups were not affected by maternal HgCl2 exposure. Dams, but not pups, presented a significant increase of Hg levels in cerebrum and cerebellum as consequence of HgCl2 exposure. The Cu levels in cerebrum of dams, as well as, in cerebellum of dams and pups were not affected by HgCl2 exposure. However, Cu levels were significantly lower in cerebrum of pups. The exposure to HgCl2 did not alter the Zn levels in cerebrum and cerebellum of dams and pups. The present study showed that the exposure to HgCl2 during lactation period increases Hg levels in cerebrum and cerebellum of dams with no alteration on the AChE activity. Of particular importance, no changes in Hg levels as well as in the AChE activity in cerebrum and cerebellum of pups exposed to Hg via maternal milk were observed in this study.

Mercuric chloride exposure during lactational period increases mercury levels in cerebrum and cerebellum of lactating rats without affecting acetylcholinesterase activity

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Abstract

The objective of this study was to investigate the effects of maternal exposure to mercuric chloride ($HgCl_2$) during mid-lactation period on acetylcholinesterase (AChE) activity from cerebrum and cerebellum of lactating rats (dams) and their pups. Dams were subcutaneously exposed to saline or $HgCl_2$ (5 mg/kg) from 8^{th} to 12^{th} day of lactation. $HgCl_2$ exposure induced a significant loss of body weight in dams and a lower body weight gain in their pups (indirectly exposed to Hg via maternal milk) without change absolute cerebrum and cerebellum weights. On the other hand, cerebrum-to-body weight ratio was significantly increased in dams and pups exposed to $HgCl_2$ whereas cerebellum-to-body weight ratio was significantly increased only in dams exposed to $HgCl_2$. AChE activity from cerebrum and cerebellum of dams and pups was not affected by maternal $HgCl_2$ exposure. Dams, but not pups, presented a significant increase of Hg levels in cerebrum and cerebellum as consequence of $HgCl_2$ exposure. The Cu levels in cerebrum of dams, as well as, in cerebellum of dams and pups were not affected by $HgCl_2$ exposure. However, Cu levels were significantly lower in cerebrum of pups. The exposure to $HgCl_2$ did not alter the Zn levels in cerebrum and cerebellum of dams and pups. The present study showed that the exposure to $HgCl_2$ during lactation period increases Hg levels in cerebrum and cerebellum of dams with no alteration on the AChE activity. Of particular importance, no changes in Hg levels as well as in the AChE activity in cerebrum and cerebellum of pups exposed to Hg via maternal milk were observed in this study.

Keywords: mercuric chloride, acetylcholinesterase, cerebrum, cerebellum, lactation and pups.

1. Introduction

Mercury (Hg), a nonessential metal, is an important environmental toxicant since it can cause severe damage to health of both animals and humans [6]. Inorganic forms of Hg (e.g. mercuric chloride – $HgCl_2$) are mainly recognized for their nephrotoxic effects [6, 12, 13, 22, 31, 32]. However, it is known that inorganic mercury (like elemental and organic forms) also is a powerful neurotoxic agent and could affect brain development, resulting in neuromorphological, neurophysiological and neurochemical effects [5, 9, 26, 46].

The pregnant and lactating women, developing fetuses and breast-fed infants are groups particularly vulnerable to mercury exposure. Experimental studies have demonstrated that exposure to inorganic mercury during gestation [44], lactation [16] or on the first days of postnatal life [14, 35, 38] induces alterations on central nervous system (CNS). Furthermore, inorganic mercury has been reported to impair the blood-brain barrier (BBB) [41, 42].

The maturation of rodents' brain occurs during several weeks of postnatal life, in which the brain is extremely sensitive to developmental disruption by chemical or environmental agents that can induce the appearance of adverse effects on postnatal development of the offspring [21]. The high sensibility seems to be due to the fact that rodents present an accelerated growth and development of organs, including the brain, during this phase. The cerebral postnatal period is divided into three phases, being the second, which ranges from 8^{th} to 12^{th} day old, the most sensitive, since it is characterized by rapid protein, DNA and RNA synthesis [21]. Besides, the BBB is not fully developed during early postnatal period in mammals; hence toxicants present in the milk could reach the CNS of pups interfering with their development [1, 2, 37]. Of particular importance, Oskarsson et al. [29] reported significant correlations between Hg levels in blood and breast milk of lactating women as a result of absorption from dental amalgam fillings. In addition, it was previously demonstrated that inorganic Hg is efficiently excreted into milk of experimental animals [43, 48].

The postnatal development of rat brain includes the maturation of cholinergic neurotransmitter system. Acetylcholine (ACh) levels and other cholinergic neurochemical markers increase markedly during development [25]. There is also evidence to suggest that cholinergic processes may be involved in the organization of

extra cellular matrix-cell-surface interactions involved in morphogenetic movements during development [11, 30]. The enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) appears to participate in neural proliferation and synaptogenesis in the developing nervous system [27]. This enzyme promotes the hydrolysis of the neurotransmitter ACh, resulting in the end of the transmission of nervous impulse in the synapses. The activity of this enzyme has been used as an index of cholinergic function, since changes in its activity can indicate alterations in the availability of acetylcholine [45]. The AChE activity can be inhibited by different toxic agents such as pesticides [4, 45], nicotine [24] and heavy metals such as mercury [9; 17, 18, 20, 28, 40]. Some authors have demonstrated the inhibition of AChE activity in brain structures of young rats exposed to $HgCl_2$ during early stages of postnatal development. Lakshmana et al. [26] showed that AChE activity was decreased in visual cortex and hippocampus from 20-day old rats chronically exposed to $HgCl_2$ (4 mg/kg bw) from postnatal day 2 by gastric intubation. More recently, Franciscato et al. [14] demonstrated that exposure to $HgCl_2$ (5 mg/kg bw) by subcutaneous injections from postnatal day 8 to 12 inhibits cerebellum AChE activity on 13-day old rats.

Considering that inorganic mercury is efficiently excreted into maternal milk and could affect the CNS development, the aim of this study was to investigate the effects of maternal exposure to $HgCl_2$ during mid-lactation period on AChE activity in cerebrum and cerebellum of lactating rats and their pups.

2. Materials and methods

2.1. Chemicals

Mercuric chloride ($HgCl_2$), sodium chloride (NaCl), potassium phosphate monobasic (KH_2PO_4) and dibasic (K_2HPO_4), tris (hydroxymethyl) aminomethane, ether, sucrose, sodium hydroxide (NaOH), 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB), nitric acid (HNO_3), sulfuric acid (H_2SO_4), o-phosphoric acid, perchloric acid, glacial acetic acid and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). Acetylthiocoline iodide, bovine serum albumin and Coomassie brilliant blue G were obtained from Sigma (St. Louis, MO, USA).

2.2. Experimental animals

Pregnant female *Wistar* rats obtained from the Animal House of the Federal University of Santa Maria were transferred to our breeding colony and maintained on a 12-h light/dark cycle and at a controlled temperature (22 ± 2ºC). The pregnant rats were housed in individual standard polypropylene plastic cages (41 x 34 x 18 cm) and allowed to deliver and wean their pups until 13th day of postnatal life. One day after the birth, litters were culled randomly to eight pups each. The animals had free access to water and commercial food (GUABI, RS, Brazil). The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Exposure to mercuric chloride ($HgCl_2$)

Twelve lactating rats were randomly divided into two experimental groups (control and $HgCl_2$) of six animals each. The exposure protocol is given bellow:

Control group: lactating rats were daily injected with physiological saline (NaCl 150 mM) from 8th to 12th day of lactation.

$HgCl_2$-group: lactating rats were daily exposed to $HgCl_2$ 5 mg/kg during the same period above mentioned.

Mercuric chloride was dissolved in physiological saline. The solutions were subcutaneously injected at a volume of 1 mL/kg body weight (bw). The animals were weighed immediately before exposure to adjust the dose. The dose of $HgCl_2$ (5 mg/kg bw) and the period of lactation which dams were exposed to mercury (from 8th to 12th day of lactation) were based on previous studies from our group [14, 15, 31-35]. Suckling pups were exposed to mercury exclusively through maternal milk. Pups body weight was daily recorded during the exposure period. All animals were observed daily throughout the study for mortality and signs of toxicity.

2.4. Tissue preparation

Twenty-four hours after the last dose of exposure all animals were weighed, anesthetized with ether and euthanized by decapitation. Cerebrum and cerebellum of lactating and suckling pups were removed, weighed and quickly placed on ice. After that, cerebrum and cerebellum were homogenized (1:10, w/v, in Tris-HCl buffer 10 mmol/L, pH 7.2 with sucrose 160mmol/L) and centrifuged (1000 x g for 15 min at 4ºC).

The supernatant fraction was used in the enzyme assay. For determination of Hg, Zn and Cu levels, the tissues were placed into vials and then frozen (-20ºC) until analysis.

2.5. Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity was determined following Ellman's method [10], modified as described in Pereira et al. [36]. The mixture assay contained 1.04 mM DTNB, 24 mM potassium phosphate buffer pH 7.2 and 25 μL of enzymatic material. It was pre-incubated for 2 min at 30ºC and the reaction was started with the addition of 0.83 mM acetylthiocoline (AcSCh) iodide. The product from the reaction of thiocholine with DTNB was determined at 412 nm every 30 sec during 2 min with an absorption coefficient of 0.0136 M^{-1} cm^{-1}. The specific activity was expressed as μmol AcSCh hydrolyzed/h/mg protein. All samples were run in triplicate.

2.6. Protein determination

The protein content was quantified by the method of Bradford et al. [3], using bovine serum albumin as a standard. All samples were run in triplicate.

2.7. Analysis of the metals

2.7.1. Digestion procedure

About 250 mg of each sample was weighed and transferred to the quartz vessels. Concentrated HNO_3 (6 mL) was added to each vessel, which was capped and placed into the microwave oven. Samples were digested using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280°C, Anton Paar, Graz, Austria). The microwave oven operated at 1400 W for 30 min and at 0 W for 20 min. The last step was used for cooling. After digestion samples were diluted with water to 25 mL and transferred to graduated polypropylene vials. Colorless and clear solutions were obtained after the digestion step. Spike recovery tests and biological certified reference material (SRM NIST 1577, bovine liver) were carried out to validate the results. Blanks were run and analyzed after each ten measurements in order to check eventual memory effects for all elements.

2.7.2. Quantification of the metals

Metal analyses were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes (for Zn and Cu). A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for Hg determinations. A deuterium background corrector was used for all the determinations. Hollow cathode lamps used were operated at 3, 4 and 4 mA, for Cu, Zn and Hg, respectively. Wavelength was set at 324.8, 213.9 and 253.7 nm and the spectral band pass at 1.2, 0.5 and 0.5 nm, respectively. Integrated absorbance (peak area) was used for all measurements. Heating program for Cu and Zn was carried out according to the recommendations of manufacturer. For Hg determination, 3 M HCl and 0.25% m/v $NaBH_4$ solutions were used as acid medium and reductant, respectively. Argon was used as purge gas. Results for all elements determined were periodically evaluated by measurements of analytical standards (each 10 measurements) and also by the digests analysis of certified reference material SRM NIST 1577 (each 3 h of measurements). If the result for standards checking presented a bias higher than 5% a recalibration procedure was performed.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. The litter was used as the experimental unit. Results were analyzed by one or two-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's t-test when appropriate. Dams and pups were independently analyzed. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. General toxicity

No maternal mortality was observed during $HgCl_2$ exposure period (data not shown). The effects of $HgCl_2$ exposure on body weight gain were analyzed by two-way ANOVA (2 exposure x 6 days) and are illustrated in figures 1A (lactating dams) and 1B (pups). For lactating dams the statistical analysis revealed significant effect of days [$F(5,50) = 6.00$, $p<0.001$] and exposure x days interaction [$F(5,50) = 9.16$, $p<0.001$]. The interaction was significant since lactating rats exposed to $HgCl_2$ presented body weight loss during exposure period. Post-hoc analyses revealed significantly reduced

maternal body weight in the $HgCl_2$-exposed group on days 11, 12 and 13 of lactation when compared to the control group (Fig. 1A).

For pups, statistical analysis revealed significant effect of exposure [$F(1,10) = 16.24$, $p<0.002$], days [$F(5,50) = 235.47$, $p<0.001$] and exposure x days interaction [$F(5,50) = 54.04$, $p<0.001$]. Although control and Hg-pups gained body weight during exposure period, the interaction was significant since Hg-pups presented a lower body weight gain than control pups. The differences between groups were verified on days 10 to 13 of lactation (after 2nd dose of $HgCl_2$ until the end of experimental period) (Fig. 1B).

3.2. Cerebrum and cerebellum weights

The $HgCl_2$ exposure did not alter absolute cerebrum and cerebellum weights of dams and pups. However, cerebrum-to-body weight ratio was significantly increased in dams (about 23%) [$t(10)= -3.218$; $p=0.017$] and pups (about 22%) [$t(10)= -4.231$; $p=0.002$] and cerebellum-to-body weight ratio was significantly increased only in dams (about 22%) [$t(10)= -2.252$; $p=0.048$] exposed to $HgCl_2$ (Table 1).

3.3. Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity of cerebrum (Fig. 2A) and cerebellum (Fig. 2B) from dams and pups were not affected by maternal exposure to $HgCl_2$ during lactation.

3.4. Metal concentration in tissues

The dams exposed to $HgCl_2$ presented higher Hg levels in the cerebrum [$t(5)= -5.947$, $p=0.018$] (Fig. 3A) and cerebellum [$t(4)= -25.604$, $p=0.001$] (Fig. 4A) than those exposed to physiological saline. The Hg levels in the cerebrum (Fig. 3A) and cerebellum (Fig. 4A) of pups were similar between control and $HgCl_2$-exposed groups.

The Cu levels in the cerebrum and cerebellum of dams were similar between control and $HgCl_2$-exposed groups (Fig. 3B and 4B). The Cu levels were significantly lower in the cerebrum of pups indirectly exposed to $HgCl_2$ via maternal milk when compared to pups of the control group [$t(4)= 12.144$, $p=0.003$] (Fig. 3B). The Cu levels in cerebellum of pups were similar between control and $HgCl_2$-exposed groups (Fig.

4B). The Zn levels in the cerebrum and cerebellum of dams and pups were similar between control and $HgCl_2$-exposed groups (Fig 3C and 4C).

4. Discussion

The present study investigated the effects of maternal exposure to a high dose of $HgCl_2$ (5 mg/kg bw) during a mid-lactation period (from 8^{th} to 12^{th} day of lactation) on acetylcholinesterase (AChE) activity in cerebrum and cerebellum of lactating rats and their pups. Our results demonstrated that $HgCl_2$ exposure during lactation period increases Hg levels in cerebrum and cerebellum of dams, but not of pups. In addition, it was demonstrated that cerebrum and cerebellum AChE activity of dams and pups were not affected by maternal exposure to $HgCl_2$ during this period.

This study revealed that lactation exposure to $HgCl_2$ during early postnatal period caused a lower body weight gain without affect absolute cerebrum and cerebellum weights on 13-day old rat pups (24h after last dose). The increase in cerebrum-to-body weight ratio observed in pups exposed to mercury through maternal milk was assumed as a consequence of reduced pup weight at end of exposure period. Our findings indicated that cerebrum and cerebellum seem to be protected against harmful effects of $HgCl_2$ during brain growth spurt (BGS), period in which a major increase in brain weight occurs [8, 47] and characterized by substantial development and proliferation of astroglial and oligodendroglial cells, synaptogenesis, and dendritic arborization [23, 47]. The mammalian BGS occurs at different times across species and is dependent upon many factors. The human BGS occurs in the second trimester of gestation, peaks at birth, and tapers at about 2 years of age. In contrast, the rat BGS occurs completely after birth with the peak occurring around postnatal day 10 [8].

$HgCl_2$-exposed dams presented body weight loss from lactation day 11 (after 3^{rd} dose of mercury). Moreover, the exposure to inorganic mercury caused an increase in cerebrum and cerebellum-to-body weight ratios. The decrease in body weight was similar to that published previously [13]. In that study it was demonstrated that body weight changes are a result of the reduced food intake, which was observed in adult female rats exposed to $HgCl_2$. In fact, the anorexigenic effects of mercury have been well documented in the literature [7, 19]. Since maternal toxicity may indirectly interfere with the development of their offspring [39], the lower pup body weight gain

observed in the present study may be related with this finding. As observed for pups, the increase in relative cerebrum and cerebellum weight were likely to be a consequence of reduced body weight of lactating dams at the end of exposure period.

The effects of $HgCl_2$ exposure on the AChE activity from cerebrum and cerebellum of lactating dams and their pups were investigated in this study. AChE is an important enzyme of cholinergic system responsible by terminus of acetylcholine action due to its hydrolysis [45]. AChE is used as a biomarker of the cholinergic function, since its activity is inhibited by different toxic agents, such as pesticides [4, 45] and heavy metals [17, 18]. In this study it was verified that the exposure of lactating dams to high dose of $HgCl_2$ during the lactation period did not affected cerebrum and cerebellum AChE activity in dams and their pups. The absence of $HgCl_2$ effect on cerebrum and cerebellum AChE activity differs from the previous results obtained by our research group. Recently, it was demonstrated that exposure to $HgCl_2$ from 8^{th} to 12^{th} day of postnatal life causes cerebellum AChE inhibition and behavioral impairments on young rats [14]. Furthermore, Sood et al. [40] and El-Demerdash [9] demonstrated cerebrum AChE inhibition in rats exposed to methylmercury and to inorganic mercury, respectively. We must stress that, in most published studies, experimental models are different from those used in present study, either in the period of development and animal species chosen or in the chemical form of mercury and route of administration utilized and, more importantly, in the physiological condition in which female rats were at the time of exposure, the lactation period.

One possible explanation for the differences observed in these studies regarding the effects of $HgCl_2$ on AChE activity could be related to Hg levels detected in the CNS. In the present study, increases in Hg levels were observed in cerebrum and cerebellum of lactating dams exposed to $HgCl_2$. Nevertheless, the degree of increase of Hg levels observed in cerebrum (~0.64 μg/g) and cerebellum (~0.59 μg/g) of lactating dams exposed to $HgCl_2$ seems to be insufficient to cause changes on AChE activity. Indeed, Franciscato et al. [14] demonstrated an inhibition on enzyme activity only when the levels of Hg were higher to 1.20 μg/g. On the other hand, pups exposed to $HgCl_2$ via maternal milk did not present increases in Hg levels in both structures analyzed. It is important to mention that the transport of inorganic mercury into milk was previously demonstrated in rats [43] and in guinea pigs [48]. In addition, we have previously demonstrated that pups exposed to $HgCl_2$ via maternal milk (same schedule of

exposure) presented increases in the kidney [13] and liver Hg levels. The specific causes for these differences are not understood and further studies are necessary.

In summary, our results represent an important finding because they demonstrate, for the first time, that exposure of lactating dams to high doses of $HgCl_2$ during the lactation period increases Hg levels in cerebrum and cerebellum without affecting AChE activity in these structures. Moreover, Hg-intoxication of dams was unable to affect CNS development of pups regarding cholinergic system.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements:

Financial support-provided by FINEP research grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net)’’ #01.06.0842-00. M.E.P. (307814/2008-4) and A.M.F. (142297/2006-2) are recipients of CNPq fellowships; C.F. is recipient of CAPES fellowships.

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Legends

Figure 1. Body weight (g) of dams (a) and pups (b) exposed to $HgCl_2$ 5 mg/kg or saline from 8^{th} to 12^{th} day of lactation. Data are expressed as mean ± S.E.M. (n = 6). * Significantly different from control group at the same day of $HgCl_2$ exposure ($p<0.05$; Student's t-test).

Figure 2. Cerebrum (a) and cerebellum (b) acetylcholinesterase activity of dams and pups exposed to $HgCl_2$ 5 mg/kg or saline from 8^{th} to 12^{th} day of lactation. Data are expressed as mean ± S.E.M. (n = 6).

Figure 3. Mercury (a), cooper (b) and zinc (c) concentrations in cerebrum of dams and pups exposed to $HgCl_2$ 5 mg/kg or saline from 8^{th} to 12^{th} day of lactation. Data are expressed as mean ± S.E.M. (n = 6). * Significantly different from control group ($p<0.05$; Student's t-test).

Figure 4. Mercury (a), cooper (b) and zinc (c) concentrations in cerebellum of dams and pups exposed to $HgCl_2$ 5 mg/kg or saline from 8^{th} to 12^{th} day of lactation. Data are expressed as mean ± S.E.M. (n = 6). * Significantly different from control group ($p<0.05$; Student's t-test).

Table 1. Cerebrum and cerebellum weights of dams and pups exposed to $HgCl_2$ 5 mg/kg or saline from 8th to 12th day of lactation

Parameters	Control	$HgCl_2$
Dams		
Cerebrum		
Absolute (g)	1.288 ± 0.027	1.327 ± 0.024
Relative (%)	0.393 ± 0.010	0.483 ± 0.026*
Cerebellum		
Absolute (g)	0.292 ± 0.019	0.302 ± 0.007
Relative (%)	0.089 ± 0.006	0.109 ± 0.007*
Pups		
Cerebrum		
Absolute (g)	0.897 ± 0.025	0.836 ± 0.029
Relative (%)	3.539 ± 0.107	4.336 ± 0.155*
Cerebellum		
Absolute (g)	0.123 ± 0.004	0.108 ± 0.009
Relative (%)	0.485 ± 0.015	0.557 ± 0.045

Data are expressed as mean ± S.E.M. (n =6). * Significantly different from control group ($p<0.05$, Student's t-test).

Figures

Figure 1a.

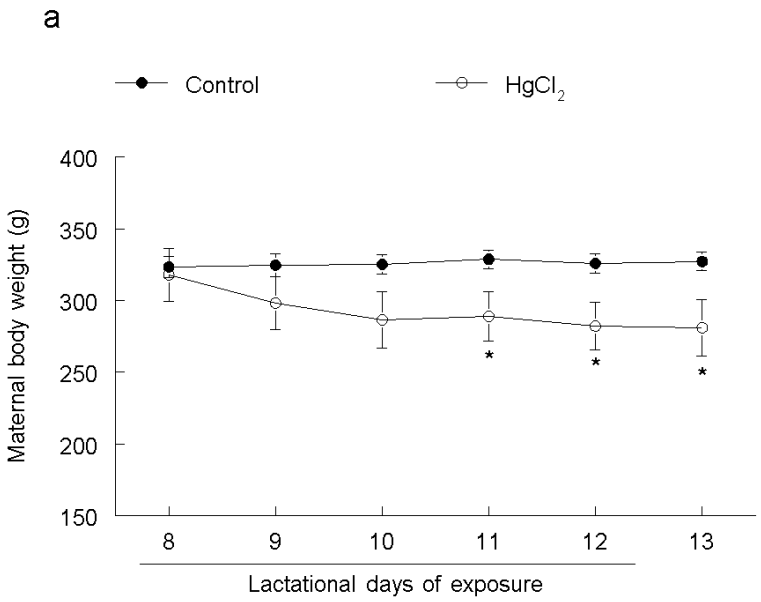
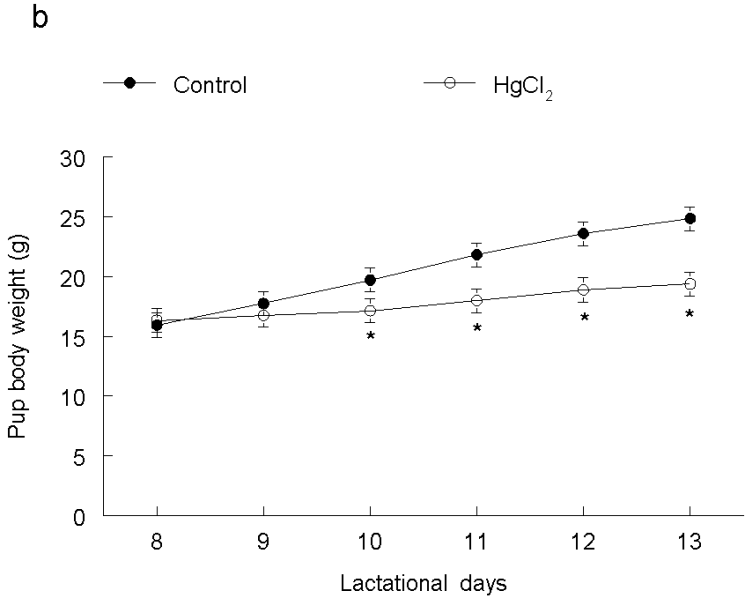


Figure 1b.



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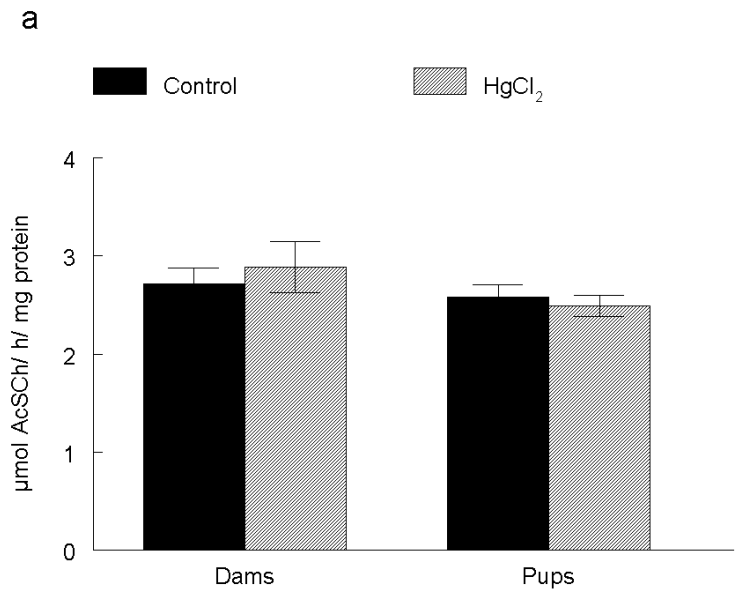

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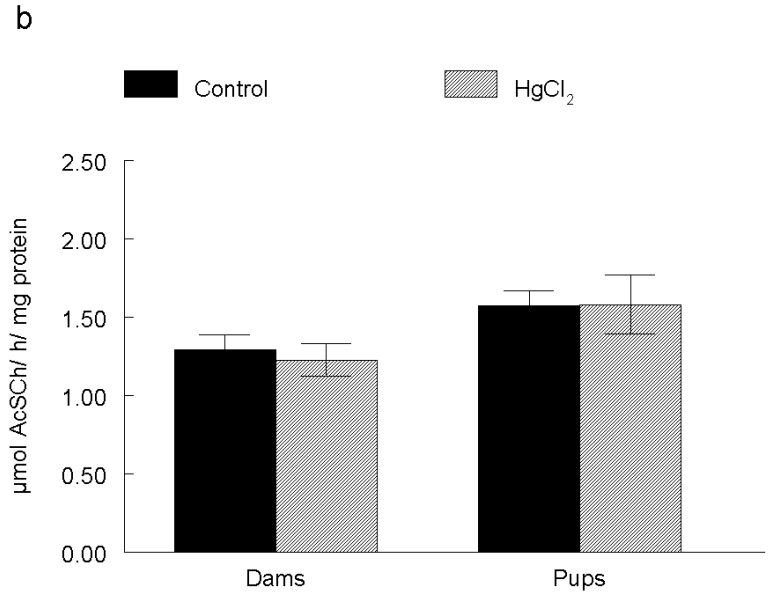


Figure 3a.

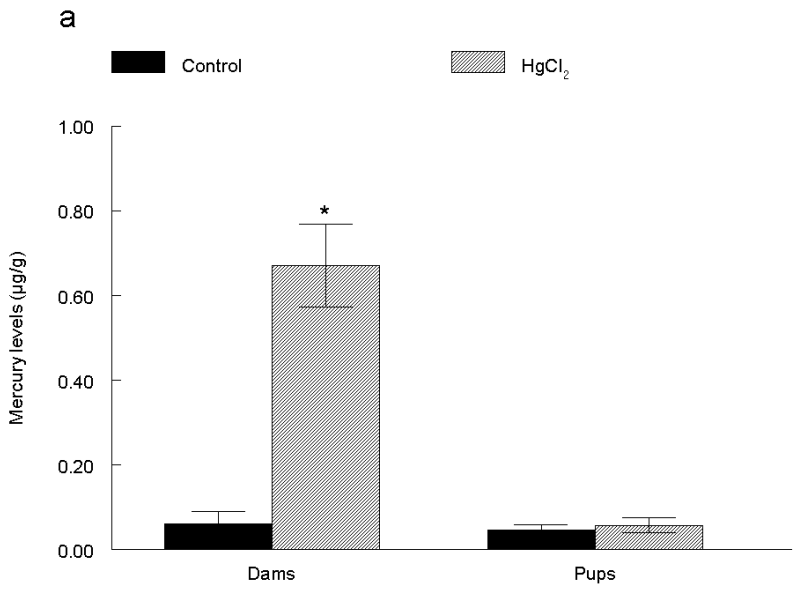


Figure 3b.

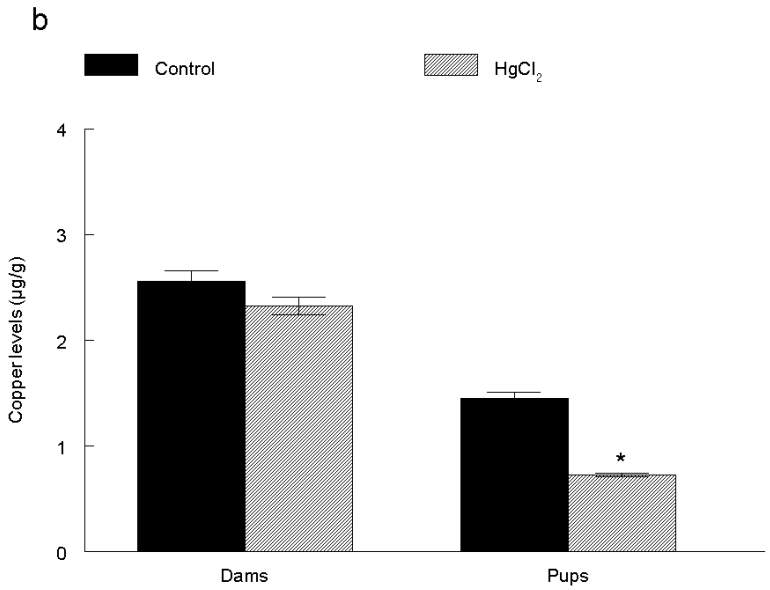


Figure 3c.

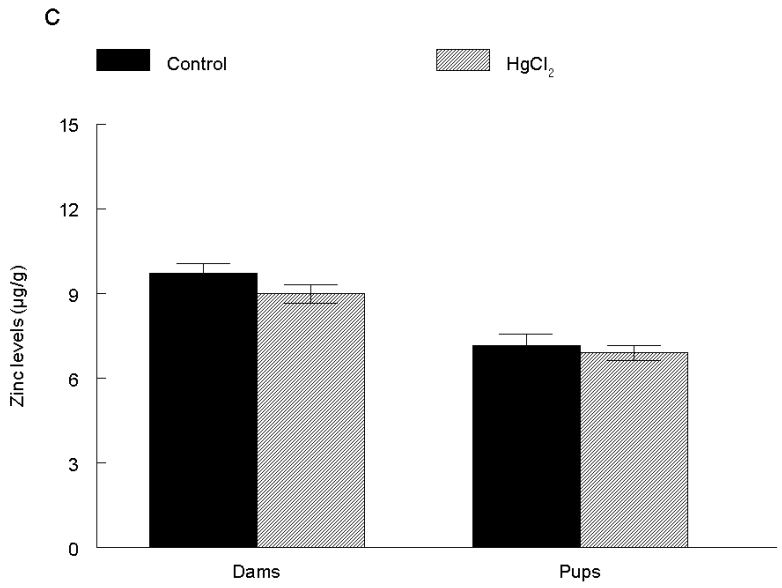


Figure 4a.

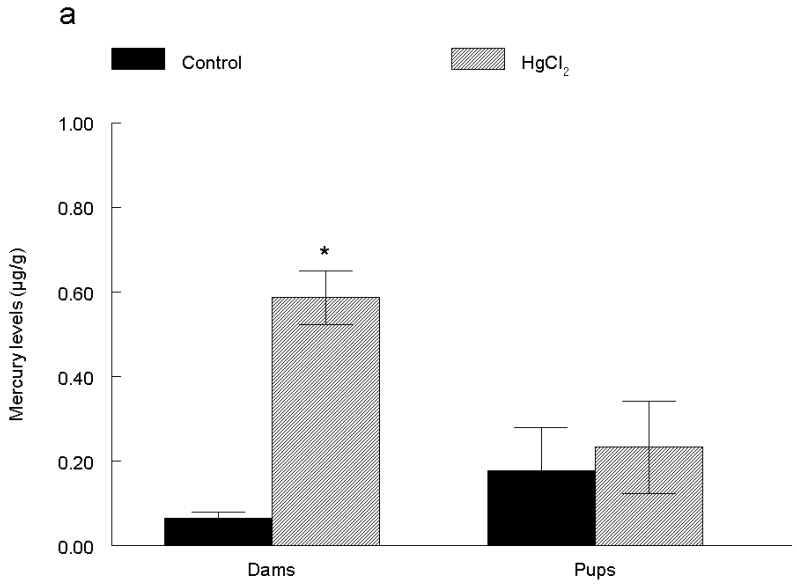


Figure 4b.

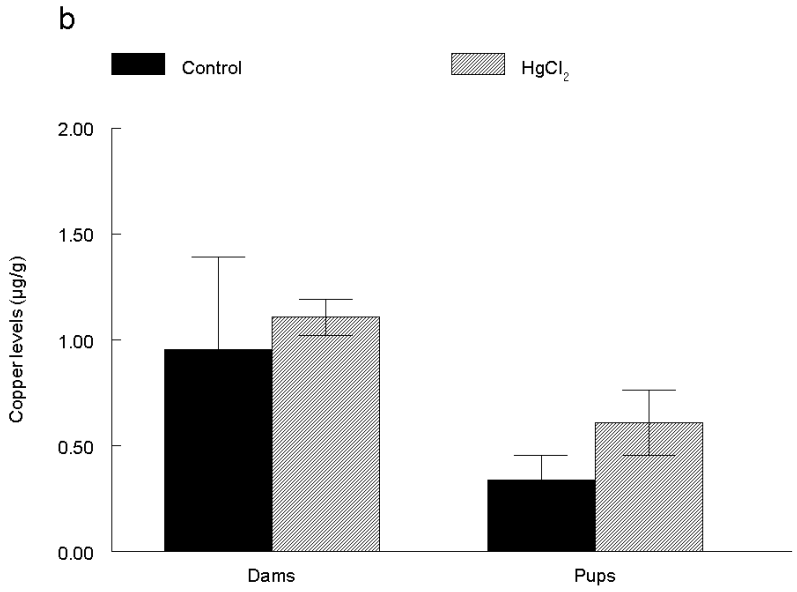
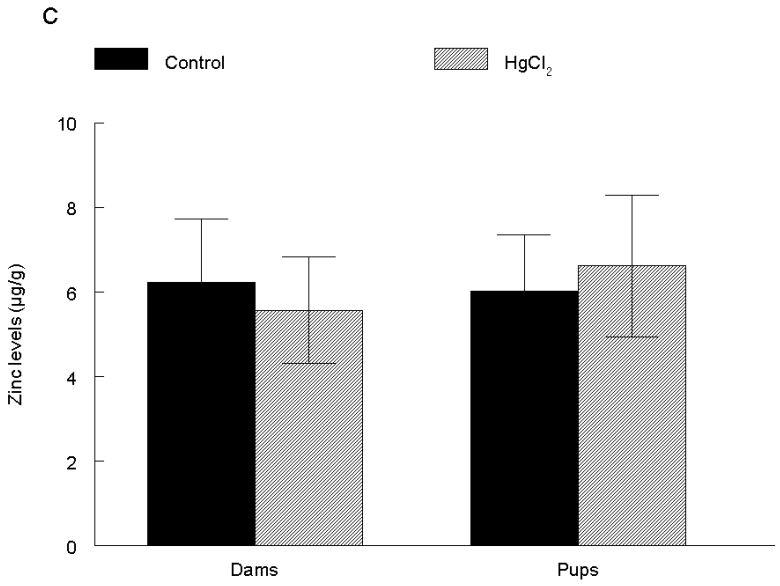


Figure 4c.



C. Artigo publicado durante a realização do curso de doutorado

Diphenyl diselenide changes behavior in female pups

Alexandre M. Favero, Simone N. Weis, Gilson Zeni, João B.T. Rocha, Cristina W. Nogueira

Artigo publicado no Periódico *Neurotoxicology and Teratology* 28 (2006) 607-616

Available online at www.sciencedirect.com
ScienceDirect
Neurotoxicology and Teratology 28 (2006) 607 – 616
NEUROTOXICOLOGY AND TERATOLOGY
www.elsevier.com/locate/neutera

Diphenyl diselenide changes behavior in female pups

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Received 31 May 2006; received in revised form 5 August 2006; accepted 5 August 2006
Available online 15 August 2006

Abstract

Diphenyl diselenide, $(PhSe)_2$, is an organoselenium compound that affects a number of neuronal processes. The effect of maternal subcutaneous (s.c.) injection of 25 mg/kg $(PhSe)_2$ once daily during early postnatal development (from PND 1 to 21) was evaluated in offspring of Wistar rats. The physical and neural reflexes were recorded at pre-weaning period. The behavioral changes in the elevated plus-maze (EPM), open-field and rotarod tasks were performed in 28-day-old pups. Selenium brain status was significantly increased (~ 41%) in rat pups. Statistically significant decreases in body weight were observed during lactation period in male and female pups exposed to 25 mg/kg $(PhSe)_2$. There were no dose-related changes on landmarks indicative of physical and reflexologic parameters of development in rats. $(PhSe)_2$ induced a disinhibitory effect in EPM behavior according to gender. Specifically, exposure to $(PhSe)_2$ increased entries and duration in the open arms of the EPM in females but not in males. Locomotor activity and rearing increased by $(PhSe)_2$ exposure in both male and female offspring in the open field. Both groups were similar in response to motor coordination in the rotarod. We concluded that maternal $(PhSe)_2$ exposure during lactation increased selenium levels in the pup brain and caused changes on developmental and behavioral parameters of Wistar rat offspring.

Keywords: Selenium; Diphenyl diselenide; Developmental; Behavioral; Rat pups; Lactation

1. Introduction

Rodents have a considerable postnatal development of the brain [12]. In fact, maturation lasts for several weeks of postnatal life, during which the brain is extremely sensitive to developmental disruption by chemical or environmental agents that can induce the appearance of adverse effects on postnatal development of the offspring. Since it is well established that fetal blood–brain barrier is not yet formed in pups at birth, drugs present in the milk would reach the offspring central nervous system (CNS), interfering with their development [1,3,28,29].

Selenium is recognized as an essential trace element and many of the lately found selenium-containing enzymes and proteins are obviously essential for normal growth, development and metabolism of an organism [5,11,14,33,34]. It becomes more and more apparent that the selenium plays a critical role in the maintenance of proper functioning of the nervous system. Selenium is a potent protective agent for neurons through the expression of selenoproteins, which are mostly involved in regulation of redox status under physiological conditions and in antioxidant defense [31]. However, despite the importance of selenium in brain physiology, it can also act as a significant environmental toxicant [9].

Diphenyl diselenide, $(PhSe)_2$, is a common intermediate in organic reactions [22,40], that displays antioxidant actions, although it is also proposed to have neurotoxic properties. Extensive studies have focused on the potential toxicological and pharmacological effects of $(PhSe)_2$ in animal models (for review, see [25]). With regard to developmental effects, it has been recently reported that single and repeated (GD6 through 15) subcutaneous administration of $(PhSe)_2$ to pregnant rats during organogenesis induces only slight embryofetotoxicity [7,39].

There is some evidence that $(PhSe)_2$ crosses the blood–brain barrier and brain selenium levels increase in mice after acute and chronic exposure to $(PhSe)_2$ [21]. Furthermore, it has been established that organoselenium compounds affect a number of neuronal processes and the exposure to high doses of these compounds causes CNS effects in mice [15,25]. Recently, our group reported that simple organoselenium compounds, including $(PhSe)_2$, present convulsant activity [24]. Likewise, it has been shown that $(PhSe)_2$ inhibits cerebral aminolevulinic

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doi:10.1016/j.ntt.2006.08.003

acid dehydratase [20] and Na^+,K^+-ATPase [4] activities in vitro. Moreover, $(PhSe)_2$ increases the basal activity of adenylyl cyclase, and inhibits [^{3}H]glutamate and [^{3}H]MK-801 binding to rat synaptic membrane preparations after both in vitro and ex vivo exposures [23]. These findings have supported the hypothesis that the brain is a potential target for the toxicity of highly lipophilic organoselenium compounds and possibly for their pharmacological and therapeutic actions.

While considerable neurotoxic effects are known about $(PhSe)_2$ exposure, little is known about the effects of developmental exposure to $(PhSe)_2$ on behavior. Therefore, the main focus of this study was to determine which effects $(PhSe)_2$ may have on offspring physical and behavioral parameters. The behavioral tests performed included anxiety in the elevated plus-maze, exploration of an open-field and motor coordination on the rotarod [16,17,27,36]. This information is very important to evaluate the safety of a possible future pharmacological application.

2. Materials and methods

2.1. Chemicals

Diphenyl diselenide (Fig. 1) was synthesized according to literature methods [26]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. This drug was dissolved in canola oil, which was obtained from a standard commercial supplier. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Pregnant Wistar rats (180–240 g) from our own breeding colony were used in this study. The animals were kept in separate animal rooms, on a 12 light/12 dark cycle, in an air-conditioned room (22±2 °C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied *ad libitum*. The pregnant rats (n=10–11/group) were assigned to two experimental groups by body weight on the day of arrival in order to achieve comparable average body weights for all groups. The dams were allowed to deliver and wean their pups until postnatal day (PND) 21. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

2.3. Treatment and maternal measures

The pregnant rats received $(PhSe)_2$ (25 mg/kg, experimental group) or canola oil (1 ml/kg, control group) once daily throughout the lactational period from PND 1 to 21 via subcutaneous injection. The dose regimen was based on LD_{50} studies from our laboratory [24]. Maternal body weight as well as food and water consumption of dams were monitored daily during the treatment period. All dams were observed daily throughout the study for mortality and signs of toxicity. At scheduled termination (PND 22), all dams were euthanized and the absolute and relative (organ-to-body weight ratio) weights of the following organs were measured: brain, liver, spleen and kidneys.

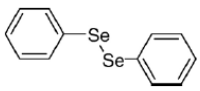

Fig. 1. Structure of diphenyl diselenide.

2.4. Offspring measures

All offspring were examined as soon as possible on the day of birth (PND 0) to determine the number of liveborn and stillborn per litter; pup health status and clinical signs were checked at least once daily throughout lactation. The viability index, as an indicator for pup mortality (pups died and cannibalized) between PND 0 and 4, and the lactation index, as an indicator for pup mortality between PND 4 and 21, were calculated. On PND 4, litters were randomly culled to four male and four female pups when possible.

2.4.1. Body and organ weights

Pups were weighed on the day after birth (PND 1) and on PND 4, 7, 14 and 21. On PND 21, pups were weaned and placed on *ad libitum* standard diets. On PND 22, randomly selected offspring (two/sex/litter) were euthanized and subjected to necropsy. The absolute and relative (organ-to-body weight ratio) weights of the following organs were measured: brain, liver, spleen, kidneys, testis (male) and uterus (female). After weaning, the animals were individually weighed. On PND 50, randomly selected offspring (two/sex/litter) were euthanized and subjected to necropsy.

2.4.2. Developmental and reflexological tests

The following parameters were recorded daily to evaluate the physical development of offspring: ear unfolding (when the tip of the ear is separated from the head), fur development (the first detection of downy hair), incisor eruption (the first appearance of upper incisors) and eye opening (when both eye lids are completely separated). One male and one female pup from each litter were marked daily with colored pens. The same male and female marked pups were employed for the observation of physical development and the mean day of appearance was calculated.

The following reflex tests were assessed once a day in the same pups: palmar grasp (grasps a paper clip with forepaws if stroked, beginning on PND 1), surface righting reflex (a normal ventral position assumed after being placed on its back for 15 s, beginning on PND2) and negative geotaxis (turns at least 180° after being placed face down on a platform inclined 45° for 30 s, beginning on PND5). The mean day of appearance of each of above parameters was calculated. Olfactory discrimination of home cage bedding was assessed on PND 14. Specifically, soiled bedding from the home cage was placed at one end of the runway (20×40×20) while fresh bedding was placed at the other end. Each pup was placed on a centerline with the head

facing the wall. Latency to reach the home bedding was recorded. If the pup did not complete the trial in 90 s or if the pup reached the clean bedding, the pup was considered to have failed the task and a maximum time of 90 s was assigned. All tests were carried out at the same time of day (8:30–10:30 a.m.), with the pups separated from the mothers at the moment of observation and then immediately returned to their home cages.

As criteria for sexual maturation the day of vaginal opening (opening of vaginal channel) for female offspring (beginning on PND 25) and testes descent (descent of both testes to scrotum) as well as preputial separation for male offspring (beginning on PND 16 and 30, respectively) was noted. For this, two male and two female pups from each litter were marked daily with colored pens. The same male and female marked pups were employed for observation and the mean day of appearance was calculated.

2.4.3. Behavioral assessments

After a 1-week post-weaning period (PND 28), the behavioral tests were conducted. All tests were carried out at the same time of day (8:30–11:30 a.m.). Rats were transported within their home cages to the test room 1 h before the tests started to minimize the influence of transportation stress. The test room temperature was 22±2 °C. The testing schedule included anxiety in the elevated plus-maze (EPM) (PND 28), exploration of an open-field (PND 29 and 30) and motor coordination on the rotarod (PND 30 and 31). The behavioral observations were blind and carried out under low-intensity light. Two male and two female per litter were randomly selected for testing of EPM, open-field and rotarod performance. The litters were tested in all behavioral tests.

2.4.3.1. Elevated plus-maze test. EPM test was employed to assess anxiety using methods similar to those previously described by [16,17,27,36]. The apparatus consisted of four arms (length: 50 cm, width: 10 cm, height from floor: 50 cm) in a cross-shaped form and a central region (10 cm^2). Two of the arms were enclosed on three sides by walls (height: 50 cm), whereas the other two were not. The enclosed or open arms of the maze faced each other. The rats were placed in the central region facing an open arm and their behavior evaluated for 5 min. We recorded the time spent in open arms and number of entries into the open and closed arms (four-paw criterion). The number of total entries (open+closed arm entries), ratio of open arms entries (open arm entries/total entries) and ratio of time spent in open arms (open arm time/total time) were calculated. The rearing (count of number that the animal stood on its hind legs) was also registered. As in all tests of exploration, the apparatus was wiped with a damp cloth and dried before the introduction of the next rat.

Table 1
Maternal parameters in rats exposed to $(PhSe)_2$ during lactation

	Control	$(PhSe)_2$ treatment
No. of litters	11	10
No. of fetuses per litter	9.73±0.62	10.20±0.93
Viability index (%)	95.24±2.13	98.75±1.27
Lactation index (%)	100.00±0.00	99.29±0.72
Body weight gain (g)		
PND 1	252.91±7.97	257.14±6.48
PND 4	259.45±7.69	249.26±7.73*
PND 7	265.36±8.39	252.76±8.06*
PND 14	276.45±8.75	264.26±8.53*
PND 21	283.00±9.42	278.92±7.87
Food consumption (g/b.w./day)		
Week 1	13.83±0.42	10.98±0.49*
Week 2	20.89±0.57	18.14±0.46*
Week 3	25.06±0.64	23.24±0.54*
Fluid intake (ml/b.w./day)		
Week 1	24.43±1.30	22.17±0.79
Week 2	33.18±1.32	31.60±1.66
Week 3	44.22±2.13	40.11±1.47

Data are reported as means±S.E.M.
* Significantly different from rats exposed to vehicle, $P<0.05$.

Table 2
Organ weight at PND 22 of dams exposed to $(PhSe)_2$ during lactation

	Control	$(PhSe)_2$ treatment
Liver		
Absolute (g)	13.77±0.23	17.36±0.77*
Relative (%)	5.00±0.06	6.05±0.12*
Brain		
Absolute (g)	1.70±0.07	1.74±0.04
Relative (%)	0.60±0.02	0.61±0.02
Kidney		
Absolute (g)	1.07±0.04	1.17±0.04
Relative (%)	0.38±0.01	0.42±0.01
Spleen		
Absolute (g)	0.49±0.04	0.70±0.04*
Relative (%)	0.17±0.01	0.25±0.01*

Data are reported as means±S.E.M.
* Significantly different from rats exposed to vehicle, $P<0.05$.

2.4.3.2. Open-field test. Spontaneous motor activity was measured in the open-field test using methods similar to those previously described by [36]. The open field was made of plywood and surrounded by walls 30 cm in height. After placing an animal at the center of the arena (45×45 cm, the floor was divided by black lines into nine equal squares 15×15 cm) the two classic variables of spontaneous behavior were recorded during a 4-min session per 2 days: (a) locomotor activity expressed by the number of crossed squares (four-paw criterion) and (b) exploratory activity expressed by the number of times rearing on the hind limbs. In order to evaluate intra-session habituation, crossing and rearing were scored at each 2-min interval.

2.4.3.3. Rotarod test. The rotarod test was performed on PND 30 and 31 for measuring motor coordination skills with similar methods as those described previously [16,17,36]. The rotarod consisted of a wooden beam covered with masking tape (diameter: 7.5 cm, length: 30 cm), used for increasing the roughness of the texture and thereby providing a firm grip. The rod was flanked by two cardboard plates in order to prevent any escape and suspended at a height of 30 cm above the mat-covered table. The rat pups were placed on top of the already revolving beam (10 rpm) and facing away from the experimenter's view in the orientation opposite to that of the beam movement in the longitudinal axis, so that forward locomotion

was necessary in order to avoid a fall. Latencies to first fall and number of falls were recorded (time limit was 180 s). Two consecutive sessions (with an inter-trial of 10 min) were performed in each day and the mean was taken as the score.

2.5. Selenium analysis

The brain samples for elemental selenium (Se^0) analysis were obtained from suckling pups, at postnatal day 22. Brain selenium status was determined using a Perkin-Elmer (Norwalk, CT, USA) model 3030 atomic absorption spectrometer equipped with a MHS-10 hydride generation system.

2.6. Statistical analysis

The litter was considered the unit of analysis. The body weight gain of dams during lactation was compared by two-way ANOVA (treatment × day), with day as a repeated measure. The body weight gain of pups was compared by three-way ANOVA (treatment × gender × day), with day as a repeated measure. Food and water intakes by dams during lactation were analyzed by two-way ANOVA using week as a repeated measure. To assess treatment × gender effects two-way ANOVA was used followed by Duncan's test when appropriate. For the open-field and rotarod behaviors, three-way ANOVA was used to assess treatment × gender × time period (intra-session habituation and days, respectively) effects followed by Duncan's test when appropriate. The remaining data were assessed by one-way ANOVA, followed by Duncan's test when appropriate. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Maternal measures

3.1.1. General

There were no significant specific overt signs of maternal intoxication (tremor and alopecia), following administration of 25 mg/kg $(PhSe)_2$ in the lactation period, when treated group was compared to the control group. No lethality was noted in any group. There were no significant differences in the number of neonates delivered and number of born alive. The viability and lactation indexes were not altered by 25 mg/kg $(PhSe)_2$ exposure (Table 1).

3.1.2. Maternal food and water consumption

For food consumption (g/b.w./day), the treatment [$F(1,19)=18.80$, $P<0.0005$] and week [$F(2,38)=390.27$, $P<0.00001$] main factors were significant but not the interaction [$F(2,38)=0.91$, $P=0.41$]. Post-hoc analyses revealed a significant decrease in food consumption in weeks 1, 2 and 3 of lactation in $(PhSe)_2$-treated group when compared to the control group (Table 1).

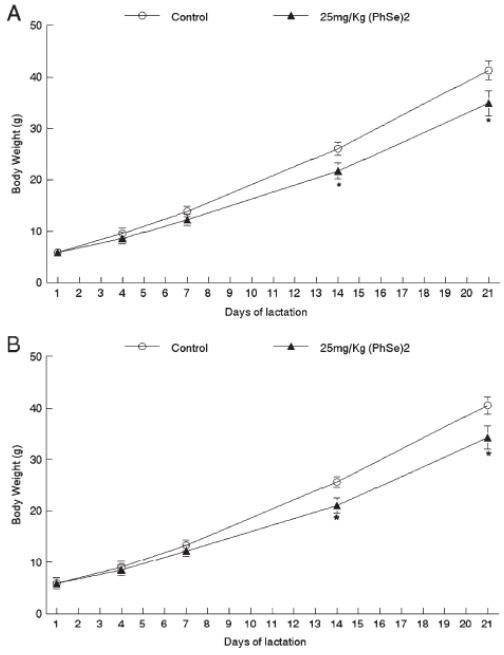

Fig. 2. Body weight (g) of male (A) and female (B) pups throughout the lactation period. Mothers were daily injected with $(PhSe)_2$ (25 mg/kg) or vehicle (canola oil) subcutaneously from PND 1 to 21. Data are expressed as mean±S.E.M. of 10–11 litters per group. *$P<0.05$.

Week factor [$F(2,38)=117.93$, $P<0.00001$], without interaction with treatment factor [$F(2,38)=0.57$, $P=0.57$], showed statistically significant alterations in water intake (ml/b.w./day) at lactation (Table 1).

3.1.3. Maternal body and organ weights

Two-way ANOVA for maternal body weight gain during lactation presented significant effects in day factor [$F(4,76)=46.94$, $P<0.00001$] and interaction between day×treatment factors [$F(4,76)=4.53$, $P<0.005$)]. Post-hoc analyses revealed significantly lower body weight in the $(PhSe)_2$-treated group on PND 4, 7 and 14 when compared to the control group (Table 1).

Significant alterations in organ weights were observed on PND 22 (Table 2). The liver and spleen weights (absolute and relative) were increased in the 25 mg/kg $(PhSe)_2$-treated group ($P<0.05$). No significant differences were observed in absolute or relative kidney and brain weights between $(PhSe)_2$ and control groups.

3.2. Offspring measures

3.2.1. Body and organ weights

Two-way ANOVA for offspring body weight at lactation presented significant effects in factors treatment [male, $F(1,19)=4.50$, $P<0.05$, and female, $F(1,19)=5.52$, $P<0.05$] and day [male, $F(4,76)=429.16$, $P<0.00001$, and female, $F(4,76)=483.97$, $P<0.00001$] with interaction between factors [male, $F(4,76)=4.37$, $P<0.005$, and female, $F(4,76)=5.59$, $P<0.0005$]. Post-hoc analyses revealed significantly lower body weight in the 25 mg/kg treatment group compared to the control on PND 14 and 21, in both male and female offspring ($P<0.05$) (Fig. 2A and B). There was no significant main factor effect of gender for offspring body weight during lactation [$F(1,38)=0.23$, $P=0.63$)]. There was no significant effect of treatment×gender×day [$F(4,152)=0.03$, $P=0.99$].

Significant alterations in organ weights (absolute and relative) were observed in male and female pups of $(PhSe)_2$-exposed group on PND 22 (Table 3). The liver weight parameters (absolute and relative) were increased in the $(PhSe)_2$-treated group of male and female offspring. Absolute brain and spleen weights were decreased in the $(PhSe)_2$-treated group of male and female offspring. Testis weight was decreased in male pups exposed to $(PhSe)_2$. Relative kidney, brain, spleen and testis weights were unaffected in the $(PhSe)_2$-treated group (Table 3). No significant differences were observed in absolute or relative uterus weight between $(PhSe)_2$-treated and control groups (Table 3).

There were no significant effects of $(PhSe)_2$ treatment for offspring body weight at necropsy on PND 50 [$F(1,38)=1.36$, $P=0.25$]. Two-way ANOVA presented main effect of gender [$F(1,38)=25.50$, $P=0.000011$] without interaction with factor treatment [$F(1,38)=0.29$, $P=0.59$]. Post-hoc analyses indicated

Table 3
Organ weight of 22-day-old male and female offspring postnatally exposed to $(PhSe)_2$

	$(PhSe)_2$ treatment			
	Male		Female	
	0	25 mg/kg/day	0	25 mg/kg/day
Liver				
Absolute (g)	1.74±0.09	2.12±0.12*	1.78±0.08	2.16±0.12*
Relative (%)	3.85±0.08	5.47±0.07*	4.04±0.12	5.62±0.18*
Brain				
Absolute (g)	1.37±0.04	1.23±0.02*	1.37±0.02	1.18±0.03*
Relative (%)	3.10±0.09	3.29±0.17	3.15±0.11	3.34±0.21
Kidney				
Absolute (g)	0.246±0.009	0.210±0.014*	0.250±0.008	0.214±0.017*
Relative (%)	0.550±0.014	0.560±0.010	0.582±0.016	0.591±0.016
Spleen				
Absolute (g)	0.212±0.014	0.157±0.018*	0.207±0.016	0.163±0.017*
Relative (%)	0.469±0.025	0.409±0.025	0.466±0.022	0.434±0.024
Testes				
Absolute (g)	0.120±0.005	0.098±0.006*	–	–
Relative (%)	0.260±0.006	0.263±0.008	–	–
Uterus				
Absolute (g)	–	–	0.038±0.005	0.031±0.002
Relative (%)	–	–	0.086±0.010	0.085±0.006

Data are reported as means±S.E.M.
* Significantly different from rats exposed to vehicle, $P<0.05$.

Table 4
Effects of maternal postnatal $(PhSe)_2$ exposure on landmarks indicative of physical parameters of development in rats

	Control	$(PhSe)_2$ treatment
No. of days at ear unfolding[a]		
Male	3.10±0.23	3.44±0.18
Female	3.20±0.25	3.56±0.18
No. of days at fur development		
Male	5.90±0.22	6.20±0.26
Female	5.90±0.22	6.20±0.26
No. of days at incisor eruption		
Male	10.90±0.53	11.30±0.54
Female	11.10±0.50	11.20±0.51
No. of days at eye opening		
Male	14.10±0.25	14.70±0.34
Female	14.36±0.20	14.70±0.34
No. of days at testes descent	20.14±0.40	20.40±0.87
No. of days at vaginal opening	37.56±0.77	37.86±0.81
No. of days at preputial separation	43.50±0.64	44.25±0.48

[a] Values represent mean (±S.E.M.) of age (PND) at expression of developmental landmarks for rats maternally exposed to vehicle or $(PhSe)_2$ on PND 1–21. Animals were inspected for these developmental landmarks beginning on PND 1.

Table 5
Effects of maternal postnatal $(PhSe)_2$ exposure on landmarks indicative of reflexologic parameters of development in rats

	$(PhSe)_2$ treatment	
	Control	25 mg/kg/day
Palmar grasp[a]		
Male	2.70±0.21	3.11±0.20
Female	2.90±0.28	3.22±0.22
Righting reflex		
Male	3.54±0.41	3.30±0.52
Female	3.00±0.40	4.10±0.53
Negative geotaxis		
Male	6.00±0.38	6.70±0.31
Female	6.10±0.26	6.70±0.35

[a] Values represent mean (±S.E.M.) of age (PND) at expression of neural reflexes for rats maternally exposed to vehicle or $(PhSe)_2$ on PND 1–21. Animals were inspected for these landmarks indicative of reflexologic parameters beginning on PND 1.

that males weighed significantly more than females ($P<0.05$) (data not shown).

There were no significant effects of $(PhSe)_2$ treatment on absolute and/or relative organ weights in both male and female pups on PND 50 (data not shown).

3.2.2. Developmental and reflexological tests

There was no significant difference in the age on which male and female pups displayed ear unfolding, fur development, incisor eruption or eye opening. Furthermore, no changes in indicators of the onset of sexual maturity, i.e. the time of testes descent and preputial separation in male pups or vaginal opening in female pups, were observed in $(PhSe)_2$-treated group (Table 4). There were no dose-related changes in the development of neural reflexes (palmar grasp, righting reflex and negative geotaxis) in both sexes (Table 5). In addition, the performance on the olfactory discrimination task was not affected by $(PhSe)_2$ exposure in both sexes (data not shown).

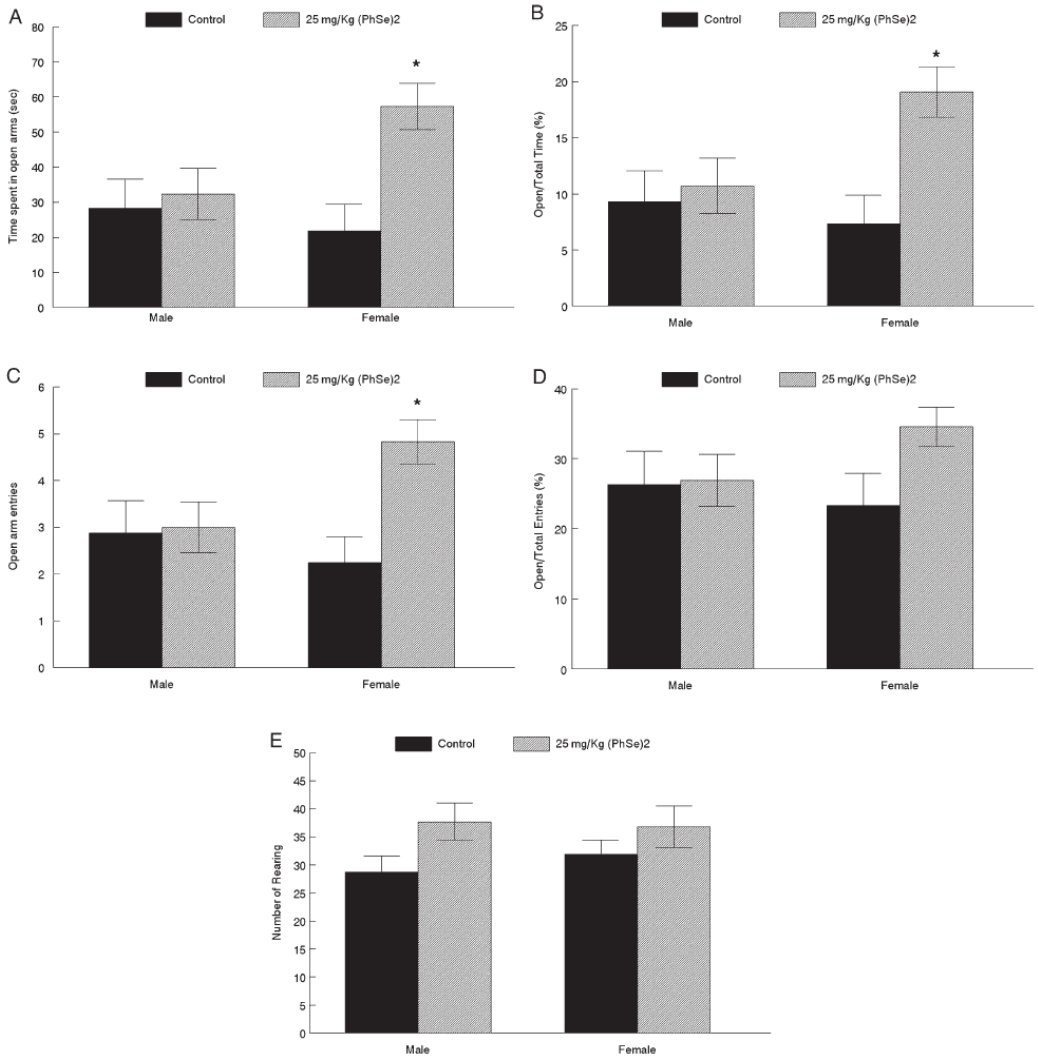

Fig. 3. Effects of postnatal exposure to 25 mg/kg $(PhSe)_2$ on: (A) time spent in open arms, (B) open/total time ratio, (C) open arm entries, (D) open/total entries ratio and (E) number of rearing in an elevated-plus maze during a 5-min test in male and female pups. Data are reported as means±S.E.M. *$P<0.05$.

3.2.3. Behavioral assessments

3.2.3.1. Elevated plus-maze test. In the EPM test, two-way ANOVA for open-arm duration and open/total duration revealed a significant main effect of $(PhSe)_2$ treatment ($[F(1,30)=6.99$, $P<0.05]$ and $[F(1,30)=6.87$, $P<0.05]$, respectively). The treatment × gender interaction terms were also significant for open-arm duration $[F(1,30)=4.24$, $P<0.05]$ and open/total duration $[F(1,30)=4.36$, $P<0.05]$. Post-hoc analyses revealed a significant increase in the time spent in the open arms and in the percentage of time spent in the open arms of the EPM only in treated female pups when compared to their respective control groups ($P<0.05$) (Fig. 3A and B).

Similarly, two-way ANOVA for the number of entries in the open arms revealed a significant main effect of $(PhSe)_2$ treatment $[F(1,30)=5.66$, $P<0.05]$ and interaction between factors treatment × gender appeared $[F(1,30)=4.66$, $P<0.05]$. Post-hoc analyses revealed a significant increase in the number of entries in the open arms of the EPM only in treated female pups when compared to their respective control groups ($P<0.05$) (Fig. 3C). There were no significant differences in number of total entries (data not shown) and in open/total entries ratio ($P>0.05$) (Fig. 3D). There was also a significant treatment main factor effect for rearing $[F(1,30)=4.64$, $P=0.05]$ (Fig. 3E).

3.2.3.2. Open-field test. Open-field behavior (number of crossing and rearing) was analyzed separately for each day. Three-way ANOVA 2 treatments (control and 25 mg/kg $(PhSe)_2$) × 2 gender (male and female) × 2 intra-session times (0–2 and 2–4 min) for the number of crossing on day 1 revealed a significant effect of treatment $[F(1,68)=5.25$, $P<0.05]$ and intra-session habituation $[F(1,68)=43.68$, $P<0.00001]$ without interaction between factors $[F(1,68)=0.71$, $P=0.40]$ (Fig. 4A and B).

Similarly, on the second day, three-way ANOVA revealed a significant effect of treatment $[F(1,68)=11.01$, $P<0.005]$ and intra-session habituation $[F(1,68)=56.84$, $P<0.00001]$ without interaction between factors $[F(1,68)=0.15$, $P=0.70]$. In day 2, post-hoc analyses revealed a significant increase in the number of crossing in the second half (2–4 min) of session in both male and female $(PhSe)_2$-treated pups when compared to their respective controls ($P<0.05$). Treatment × gender × intra-session habituation interactions were not observed for number of crossing on day 1 $[F(1,68)=0.51$, $P=0.48]$ and day 2 $[F(1,68)=0.05$, $P=0.82]$.

Regarding the number of rearing, three-way ANOVA for the day 1 revealed a significant main effect of treatment $[F(1,68)=5.25$, $P<0.05]$ and treatment × intra-session habituation interaction $[F(1,68)=43.68$, $P<0.00001]$. Post-hoc analyses revealed a significant increase in the number of rearing in

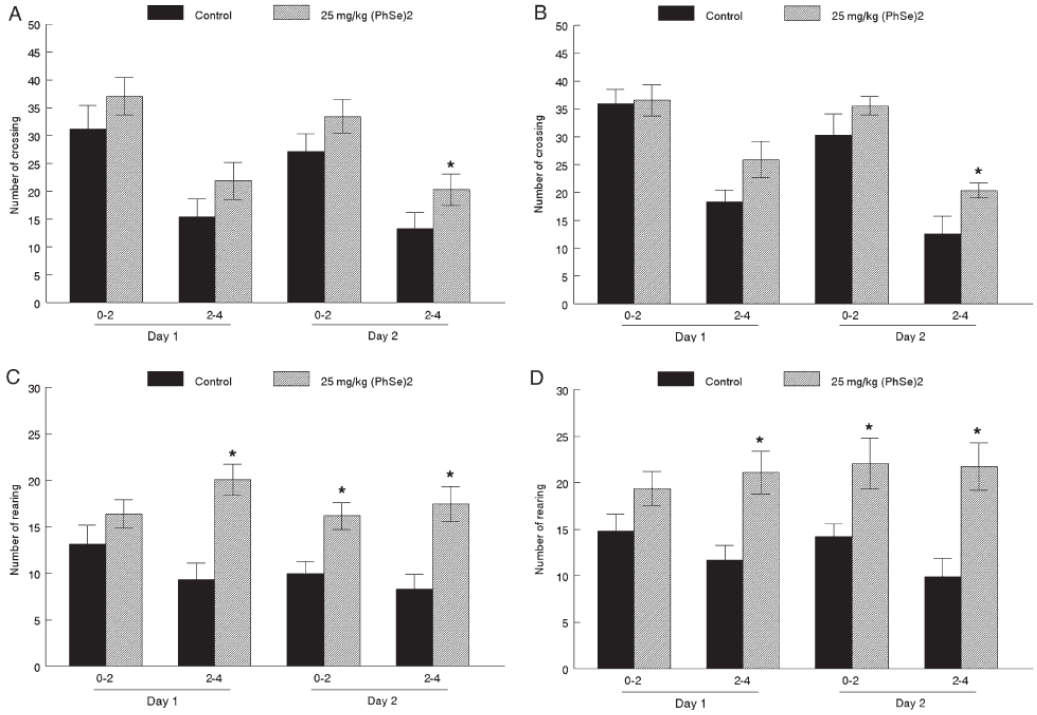

Fig. 4. Effects of postnatal exposure to 25 mg/kg $(PhSe)_2$ on the number of crossing and rearing in open-field behavior during a 4-min session (0–2 and 2–4 min) in male (A and C, respectively) and female (B and D, respectively) pups. Data are reported as means ± S.E.M. *$P<0.05$.

Table 6
Effects of maternal postnatal $(PhSe)_2$ exposure on rotarod motor coordination in rats

	Latency (s) to first fall		Number of falls	
	PND 30	PND 31	PND 30	PND 31
Males				
Control	84.93±8.16	166.85±5.97	1.72±0.31	0.14±0.08
25 mg/kg $(PhSe)_2$	96.63±9.44	165.64±8.31	1.38±0.12	0.15±0.08
Females				
Control	94.03±5.11	157.61±7.09	1.56±0.24	0.17±0.05
25 mg/kg $(PhSe)_2$	72.29±12.91	149.95±12.01	2.00±0.22	0.25±0.10

Data are reported as means±S.E.M.

the second half (2–4 min) of session in both male and female $(PhSe)_2$-treated pups when compared to their respective controls ($P<0.05$) (Fig. 4C and D).

On day 2, three-way ANOVA revealed a significant main effect of treatment [$F(1,68)=40.04$, $P<0.00001$] and gender [$F(1,68)=8.32$, $P<0.005$] without interaction between factors [$F(1,68)=0.61$, $P=0.44$]. Post-hoc tests revealed a significant increase in the number of rearing in the first (0–2 min) as well as in the second half (2–4 min) of session in both male and female $(PhSe)_2$-treated pups when compared to their respective controls ($P<0.05$). Treatment × gender × intra-session habituation interactions were not observed for number of rearing on day 1 [$F(1,68)=0.26$, $P=0.61$] and day 2 [$F(1,68)=0.04$, $P=0.83$].

3.2.3.3. Rotarod test. Three-way ANOVA 2 treatments (control and 25 mg/kg $(PhSe)_2$) × 2 gender (male and female) × 2 days (PND 30 and PND 31) for latency to first fall and number of falls in the rotarod test revealed only a significant effect of days [$F(1,36)=124.10$, $P<0.0001$] for fall time and [$F(1,36)=165.82$, $P<0.0001$] for fall number (Table 6). Rat pups from both groups increased their latencies to first fall and decreased the fall number in the second day, indicating that performance improved over 2 days of testing. Treatment × gender × day interaction was not observed for latency to first fall [$F(1,36)=1.06$, $P=0.31$] and for the number of falls [$F(1,36)=2.42$, $P=0.13$].

3.3. Selenium analysis

Exposure to 25 mg/kg $(PhSe)_2$ caused a significant increase of approximately 41% in whole brain selenium (Se^0) status in 22-day-old suckling pups. The brain selenium levels for control and $(PhSe)_2$-treated groups were 85.0±7.5 ng/g and 120.0±10.4 ng/g, respectively ($n=3$–4).

4. Discussion

The current study evaluated the effects of lactational exposure to $(PhSe)_2$, a common intermediate in organic reactions that displays promise in pharmacological applications, on developmental and behavioral measures in Wistar rats. We clearly demonstrated that $(PhSe)_2$ exposure during neonatal development increased selenium levels in the pup brain and induced a disinhibitory effect in EPM behavior according to gender. Specifically, exposure to $(PhSe)_2$ provoked an increase in the percentage of time spent in the open arms in females but not in males. In contrast, direct assessment of locomotor behaviors showed that locomotor activity and rearing behavior were increased by $(PhSe)_2$ exposure in both male and female offspring.

$(PhSe)_2$ exposure significantly decreased body weight gain in pups during the neonatal development period. There are some data suggesting that drugs that interfere with offspring growth might directly or indirectly affect subsequently maturation and development of neonates [32]. In our study, however, postnatal treatment with $(PhSe)_2$ did not affect developmental landmarks (ear unfolding, fur development, incisor eruption and eye opening) in both male and female offspring.

According to Altman and Sudarshan [2], three peripheral systems may be involved in the regulation of postural adjustments, including vestibular, extereoceptive (e.g., tactile) and proprioceptive systems. The vestibular system functions at birth although vestibular reactions are hampered by immaturity of the motor system. The righting reflex and geotaxis response of young rats reflect both motor development and activity guided by the vestibular system [2]. In this study, postnatal $(PhSe)_2$ exposure did not alter the development of these two reflexes in the male and female offspring.

Regarding maternal parameters, $(PhSe)_2$-treated dams significantly decreased food consumption and gained less weight from PND 4 to 14 than did the control group; however, the body weight was similar to the control at term of lactational period. Moreover, the exposure to $(PhSe)_2$ caused an increase in liver- and spleen-to-body weight ratios. The decrease in body weight and the increase in liver ratio were similar to those published previously [20]. Since maternal toxicity may indirectly interfere with the development of their offspring [32], the decreased pup body weight observed in the present study may be related with these findings.

In $(PhSe)_2$-treated male and female offspring, a number of organs including, brain, testes, spleen and kidney had weight changes that occurred in a pattern typically associated with decrements in body weight. For these organs, absolute weights decreased while organ-to-body weight ratios were normal. This pattern of change in weight parameters for these organs indicates that such weight effects were most likely a consequence of the body weight decrement and were not an indicative of primary target organ toxicity. In contrast, general systemic effects of $(PhSe)_2$ were evident in terms of increased liver weights (absolute and relative). In fact, the liver is vulnerable to chemical injuries due to its anatomical proximity to the blood supply and digestive tract as well as its ability to bio-transform and concentrate xenobiotics [19]. Accordingly, our group has reported that 10 mg/kg $(PhSe)_2$ administered to mothers for 2 weeks caused an increase in plasma ALT activity in pups [8]. Thus, from combined results of both studies, we could infer that the liver is the target-organ for intoxication by organoselenium compounds in rat pups. Interestingly, our results demonstrated the lack of $(PhSe)_2$ effects on body and organ weights (absolute and relative) of pups on PND 50. Based on these results, we emphasize that exposed offspring recovers to normal levels,

suggesting that the differences related to $(PhSe)_2$ exposure observed on PND22 were transient.

It has been demonstrated that chronic exposure to high doses of $(PhSe)_2$ caused a dose-dependent increase in Se deposition in brain of mice [21]. Accordingly, our group has demonstrated that maternal exposure to $(PhSe)_2$ during lactation led to an increase in selenium levels in serum of suckling pups, reinforcing that elemental selenium became bio-available to pups via maternal exposure [8]. In the present study, selenium brain status was significantly increased in rat pups. Therefore, from the results of current as well as early studies, it is evident that the maternal exposure to $(PhSe)_2$ during critical period of development led to an increase of selenium levels in the nursing pups.

The action of nervous system and its subtle disruption functioning by xenobiotics could be evaluated through the performance of animals in several behavioral tests [10,16]. The elevated plus-maze test is based on the natural aversion of rodents for heights and open spaces. This behavioral test has been pharmacologically and ethologically validated in mice [18] and rats [27], and it is widely employed in animal models to assess anxiety (for reviews, see [13,30]). The results from the present study indicated that neonatal exposure to $(PhSe)_2$ produced an anxiolytic-like response only in females, whereas it did not modify anxiety in males according to the EPM test. Increased open-arm exploration by treated female rats may be caused by disinhibitory tendencies [16,17]. From an ethological point of view, excessive disinhibitory tendencies can be not adaptative and can potentially expose the affected animal to dangerous situations, which emphasizes the potential neurotoxicity of this organoselenium compound. Recently, we demonstrated that diphenyl ditelluride, a recognized teratogen in rats and structural analogues of $(PhSe)_2$, causes neurobehavioral changes very similar to those observed in the present study in male pups exposed to this compound during the suckling period [35,36].

Exploration is a very important behavior by which the animal gains information about its environment and it is an essential life-preserving component of an animal's higher nervous functions [37]. In the present study, both developmentally selenium-exposed male and female pups demonstrated a significant increase in locomotor activity and rearing behaviors, indicating that $(PhSe)_2$-exposed offspring were more active than controls. It is well known that the interpretation of the results about anxiety effects are sometimes confounded by changed levels of motor activity [6]; however, in our study, the gender-specific differences related to $(PhSe)_2$ exposure observed in the EPM test could not be attributed to the increase in the locomotor activity, since selenium exposure during the suckling period altered both male and female exploratory behaviors in the open-field test. We have suggested that female pups appeared to be more sensitive to $(PhSe)_2$ exposure during postnatal development of the rat brain.

The selenium status in the brain during developmental period may account for alterations on behavior observed in the current study. Accordingly, Watanabe and Satoh [38] demonstrated that selenium-deficient female pups showed less movement than did the control group, in the open-field test. On the other hand, such a significant difference was not found with the males. Thus, it is reasonable to admit that there is a clear vulnerability in the female pups behavior related to alterations on selenium levels during early neonatal period.

In summary, we demonstrated that maternal exposure to $(PhSe)_2$ during lactation increases selenium levels in the pup brain and changes behavior in female pups. $(PhSe)_2$ affected the body weight gain in exposed pups indicating that, of all the developmental measures used in this study, the pup body weight was the most sensitive indicator of developmental toxicity. Further researches employing different behavioral paradigms and neurochemical parameters are warranted. The reasons for the gender difference induced by $(PhSe)_2$ in behavioral tasks remain to be elucidated, as well.

Acknowledgements

The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged. C.W.N., J.B.T.R. and G.Z. are the recipients of CNPq fellowships.

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